GATA CO-FACTORS: COLLABORATORS IN CARDIAC DEVELOPMENT, CONSPIRATORS IN CARDIAC DISEASE

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DEDICATION

To my mother and father,

Sugra and Shiraz Kathiriya.

GATA CO-FACTORS: COLLABORATORS IN CARDIAC DEVELOPMENT, CONSPIRATORS IN CARDIAC DISEASE

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2005

ACKNOWLEDGEMENTS

Similar to other complex traits, curiosity can be attributed to genetic and environmental components; thanks, mom and dad. I'd like to also thank my family, including Adam, Salim and the Adenwala's, and my friends, especially Stan, Steve and Leland, for their support.

This work could not have been accomplished without the contributions from several individuals. Special thanks to Dr. Richardson and his group for assistance with histological and in situ analysis, Dr. Robert Gerard for generating recombinant adenoviruses, Jay Hunter for accepting my DNA samples for sequencing after hours, Stephen Johnson for refined graphics preparation and many collaborators who have shared reagents.

I am thankful to the NIH Medical Scientist Training Program for their financial support, as well as Dr. Rodney Ulane, Dr. Munford, Robin Downing and Stephanie Robertson, for their moral support and professional advice. I greatly appreciate the time and effort of members of my thesis committee, Drs. Xiadong Wang, Masashi Yanagisawa, Eric N. Olson and Deepak Srivastava.

For useful insights, thoughtful critiques and largess with their resources, I would like to thank Dr. Eric Olson and members of his laboratory.

Thanks to all of the members of the Srivastava lab for their years of friendship and for making the research environment such an enjoyable place to work.

For a fruitful collaboration, I am grateful to Vidu Garg.

For patiently teaching me the scientific method, I am indebted to Osamu Nakagawa.

To my better half, in life and in lab, thank you Isabelle, for everything that I cannot put into words.

"If you prepare yourself... you will be able to grasp opportunity for broader experience when it appears" (Eleanor Roosevelt). To my mentor, Deepak, thank you for cultivating in me skills for success in and out of the lab.

GATA CO-FACTORS: COLLABORATORS IN CARDIAC DEVELOPMENT, CONSPIRATORS IN CARDIAC DISEASE

Publication No._____

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The University of Texas Southwestern Medical Center at Dallas, 2005

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Disruption of fetal gene expression during cardiac development can result in congenital heart defects (CHDs), the most common developmental anomaly in humans and the leading non-infectious cause of death in newborns. The reactivation of fetal gene expression during cardiac hypertrophy in the adult is an adaptive response to pressure or volume overloads, but can lead to impaired cardiac function. Gata4, a member of a family of zinc-finger transcription factors, has been implicated as a key regulator of fetal cardiac gene expression during cardiac

development and cardiac hypertrophy. This thesis work presents two novel transcriptional complexes likely found in these settings, one that cooperates to activate GATA-dependent transactivation and one that represses it.

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ABBREVIATIONS

αAR – α-adrenergic receptor-mediated pathway

α-MyHC – alpha myosin heavy chain

βAR – β-adrenergic receptor-mediated pathway

β-*MyHc* – beta myosin heavy chain

Ad – adenovirus

Akt/PKB – nomenclature derived as a gene first identified from a thymoma in an AKR mouse, also known as protein kinase B

Akt*– "constitutively active" form of Akt, with myristilation

ANF – atrial natriuretic factor

AngII – angiotensin II

A-P – anterior-posterior

AVC – atrioventricular canal

AVS– atrioventricular septum

BMP – bone morphogenetic protein

CaMK – Calcium/calmodulin-dependent protein kinase

CaMKIV* – constitutively active form of CaMK-IV

CHDs – congenital heart defects

CSDs – cardiac septal defects

Cxn40 – connexin 40

C. elegans – Caenorhabditis elegans

D. melanogaster – Drosophila melanogaster

dHAND – dedicidua, heart and neural crest derivatives transcription factor, also known as Hand 2

DMEM – Dulbecco Modified Eagle Medium

DNA – deoxyribonucleic acid

EMSA – electromobility shift assays

ET-1 – endothelin-1

FBS – fetal bovine seurm

Fog-2/Zfpm-2 – friend of gata-2, also known as zinc finger protein-2

Gata4(mG295S) – G295S mutation in mouse Gata4

Gata4(mE360del) – mE360del mutation in mouse Gata4

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

grl – gridlock

GSK – glycogen synthase kinase

Hand2 – heart and neural crest derivatives transcription factor-2

HDAC – histone deacetylase

hE359del – refers an inherited mutation of human GATA4 that is associated with CHDs

hG296S – refers an inherited mutation of human GATA4 that is associated with CHDs

Hrt – hairy-related transcription factor

IGF-1 – insulin-like growth factor-1

Iso – isoproteronol

Jmj – Jumonji

L-R – left-right

LV – left ventricle

M199 – Medium 199

MAPK/ERK – mitogen activated protein kinase/ extracellular signal-regulated kinase

MBP – maltose binding protein

Mef2c – myocyte enhancing factor-2c

MOI – multiplicity of infection

MyoD – myogenic differentiation factor

myr – myristilated

NCoR – nuclear receptor co-repressor

NFAT – nuclear factor in activated T-cells

NLS – nuclear localization signal

P/S – penicillin/streptomycin

PE – phenyleprhine

PCR – polymerase chain reaction

PDGF – platelet-derived growth factor

PI3K – phosphotidylinositol 3 kinase

PKA – protein kinase A

RNA – ribonucleic acid

RSK2*/MAPKAPK – Ribosomal protein S6 kinase, also known as mitogen activated protein kinase-activated protein kinase, constitutively active form

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel

SERCA2 – sarcoplasmic reticulum calcium pump-2

Smad – nomenclature derived from *sma* genes in *C. elegans* and *mad* genes in *D. melanogaster*

SRF – serum response factor

Su(H) – suppressor of hairless, also known as recombinant binding protein-Jk (RBP-Jk)

TGA – transposition of the great arteries

TGF – transforming growth factor

Wnt – nomenclature originally derived from the Drosophila *wingless* mutant and int-1 in mammals

SUMMARY

Gata4 or its orthologs are essential for cardiac development in flies, fish and mice. We recently identified *GATA4* as a causative gene for human cardiac septal defects through genetic linkage analysis of pedigrees with non-syndromic CHDs. To identify the mechanisms by which *GATA4* mutations may cause disease, constructs harboring the human mutations were generated and tested. Mutation of a highly conserved residue (G296S) of *GATA4* resulted in diminished DNA-binding affinity and transcriptional activity of Gata4. In addition, the Gata4 mutation abrogated a novel physical interaction between Gata4 and TBX5, a T-box protein responsible for a subset of syndromic cardiac septal defects. Conversely, interaction of Gata4 and TBX5 was disrupted by specific human *TBX5* missense mutations that cause similar cardiac septal defects. In a second family, a frame-shift mutation of *GATA4* (E359del) was transcriptionally inactive. In children with sporadic CHDs, we have identified several additional *GATA4* mutations. **These results implicate *GATA4* as a genetic cause of human cardiac septal defects, perhaps through its interaction with TBX5.**

In addition to its role in development, Gata4 activates fetal gene expression in the maladaptative process of post-natal cardiac hypertrophy. With others, I discovered that Hrt2 (hairy-related transcription factor-2) a cardiac transcriptional repressor, inhibited induction of the cardiac fetal gene program during

cardiomyocyte hypertrophy. A screen for candidate cardiac transcription factors that induce the fetal gene program and are sensitive to Hrt-mediated repression revealed a physical interaction between Hrt2 and Gata4 that was necessary for repression of GATA-transactivation. Since growth signals are transmitted from the cell membrane to the nucleus via second messengers, including kinases and phosphatases, nearly twenty candidate kinases and phosphates implicated in cardiac development and hypertrophy were tested for effects on Hrt-mediated repression of Gata4. Only one, Akt/PKB, was able to ameliorate Hrt-mediated repression of transactivation by Gata4, but not by Notch, a broadly-expressed transcription factor that is repressed by Hrt2. The Akt-responsive region of Hrt2 was mapped to a conserved motif and a phosphomutation of a serine residue in this domain of Hrt2 mimicked the effects of Akt on Hrt repression of Gata4, but not Notch. **These results provide evidence for a molecular pathway by which Akt modulates Hrt-mediated repression, to regulate GATA-dependent cardiac gene expression.**

INTRODUCTION
DEVELOPMENT OF THE HEART

Cardiac development is a complex morphogenetic process involving combinations of DNA binding proteins and co-factors, which form functional units that regulate distinct subsets of target genes for the precise orchestration of cell movement, cell proliferation, cell death and cell differentiation. As one of the first organs to develop, the heart is critical for pumping nutrients, including oxygen, to the growing embryo. Here, I summarize recent progress in the field regarding the developmental biology of the heart, by describing some salient aspects of embryology and molecular biology of cardiac gene expression (reviewed in Kathiriya and Srivastava, 2000).

Early Cardiogenesis

Soon after gastrulation, cardiogenic precursors from mesodermal cells in the anterior lateral mesoderm respond to opposing BMP and Wnt gradients from adjacent endoderm to form a region known as the cardiac crescent (Schneider et al., 2001; Tzahor et al., 2001). Several genetic markers are expressed in the cardiac crescent, while only a few transcription factors, including Gata4, TBX5, Mef2C and Nkx2.5, have been shown to be sufficient to induce ectopic cardiac gene expression in pluripotent P19 cells (Durocher et al., 1997; Hiroi et al., 1997; Skerjanc et al.,

1998), suggesting that these factors may have some cardiogenic potential. However, a factor that is sufficient to de-differentiate a cell to a cardiac fate is highly sought after and remains to be identified.

Cardiac progenitors are specified to inhabit distinct segments of the linear heart tube that is patterned along the anterior-posterior (A-P) axis--conotruncus (outflow tract), right (pulmonary) and left (systemic) ventricles, and atria. Although the left and right ventricular precursors are initially mixed along the A-P axis at the cardiac crescent stage, these cells assume their final Left-Right (L-R) position as a result of rightward looping of the heart tube. In contrast, the right atrium appears to develop from right atrial progenitors, while the left atrium develops from those on the left. Interestingly, mouse models mutant for *activin receptor IIb* (Oh and Li, 1997) or *lefty-1* (Meno et al., 1998) display right or left atrial isomerism, respectively, suggesting responsiveness by the left and right atria to signals that determine L-R asymmetry.

Process of Cardiac Looping

The process of cardiac looping intricately establishes the relative positions of the cardiac chambers and their vascular connections (Figure 1A-C). The inflow and outflow tract cushions, swellings of extracellular matrix that remodel into valve tissue, become positioned adjacent to one another by folding of the heart tube. Subsequently, the left and right atria must align with the appropriate ventricular

chambers, and each ventricle must connect with the aorta or pulmonary artery.

This process is mediated by morphogenesis of the atrioventricular septum (AVS), which divides the inflow tract, known as the common atrioventricular canal (AVC), into a right and left AVC. As the AVS shifts to the right to lie above the ventricular septum, the AVCs follow, consequently, to become aligned over their respective ventricles. In conjunction, the common outflow tract, known as the truncus arteriosus, septates to become the aortopulmonary trunk. As it shifts to the left to situate itself over the AVS, the vascular trunk twists 180° to position the aorta properly over the left ventricle and the pulmonary artery correctly over the right ventricle. In this manner, the cardiovascular system is converted from a circuit in series to a parallel circulation, in preparation for life away from the womb.

Cellular and Molecular Biology of Cardiac Looping

Molecular modifications in cellular proliferation, transformation, migration and death are thought to be involved in the process of looping, but the relative contributions of these cellular mechanisms remain unknown. The inner curvature of the looping heart tube appears to be remodeled, while the outer curvature actively proliferates. A phenomenon known as *myocardialization* may explain why trabeluations in the ventricles are found on the outer curvature while the inner curvature remains smooth (reviewed in Mjaatvedt et al., 1999). Along the inner curvature, cardiomyocytes evacuate and migrate to the cushions where they invade

and muscularize without proliferating, resulting in the relocation of myocardium from the inner curvature to the cushions. Mice with trisomy 16 (syntenic to parts of human chromosome 21 and 22) have defects in myocardialization, such that the myocardium of the inner curvature cannot be removed or remodeled in the absence of cushions, resulting in defects in the process of looping (Webb et al., 1996).

Genes differentially expressed along the outer or inner curvatures of the looping heart tube have been discovered and may provide a molecular basis for their differences. Genes encoding atrial natriuretic factor (ANF) and SERCA2, the sarcoplasmic reticulum calcium pump necessary for cardiac excitation and contraction, are expressed in the outer curvature of the ventricles and atria but are absent in the inner curvature (Christoffels et al., 2000). Additionally, two members of the basic helix-loop-helix family, *dHAND* and *eHAND*, are expressed in a complementary and ventricle-enriched fashion, predominantly along the outer curvature of the heart (Biben and Harvey, 1997; Srivastava et al., 1997; Thomas et al., 1998). This evidence suggests that intrinsic properties of the two surfaces may be central to cardiac looping.

Aberrations in the process of cardiac looping may result in a number of congenital heart diseases involving alignment or septal defects. After heart tube has initiated cardiac looping, any arrest or delay in the positioning of the AVS or the conotruncal region may result in malalignment of the inflow and outflow tracts with the ventricles (Figure 1D-G). For example, if the conotruncus is unable to migrate to

the left, the right ventricle communicates with the aorta and pulmonary artery, resulting in a condition known as double-outlet right ventricle (Figure 1E). Likewise, the AVS may fail to move to the right, resulting in a condition known as double-inlet left ventricle, in which the left ventricle communicates with both the left and right AVC (Figure 1F). Genetic evidece for this has been discovered from embryos containing a targeted disruption for *Fog-2/Zfpm-2* that have a single AVC, which appears to communicate with only the left ventricle (Sevnsson et al., 2000; Tevosian et al, 2000). However, the molecular basis for the shifts necessary for proper alignment remains unknown.

If the conotruncus moves appropriately to lie over the AVS but fails to twist, a condition known as transposition of the great arteries (TGA) may result (Figure 1G). In TGA, the pulmonary artery communicates with the left ventricle while the aorta communicates with the right ventricle, resulting in two separate circuits in which blood in the systemic circulation fails to be oxygenated. Molecular evidence for TGA has been gathered from embryos that are *trans*-heterozygous for mutations of *nodal* and *Smad2*, a downstream intracellular signaling component of the Nodal/TGF-β pathway (reviewed by Schier and Shen, 2000), which display laterality defects and an outflow alignment deft that is limited to TGA (Nomura and Li, 1998).

Taken together with other clinical observations, such molecular evidence hints at the interaction of pathways that regulate the process of looping as well as the directionality of looping. Indeed, some matrix proteins in the chick, such as

flectin (Tsuda et al., 1998), are expressed asymmetrically along the linear heart tube and may play a role in the mechanical forces guiding cardiac looping. The discovery that *Pitx2* is expressed asymmetrically in the lateral plate mesoderm and on the left side of developing organs (Piedra et al., 1998; Logan et al., 1998; Yoshioka et al., 1998; Ryan et al., 1998) provides the first transcriptional inroad into understanding how L-R signals might affect organogenesis. In the heart, targets of Pitx2 on the left side of the heart tube may interact with cascades governing cardiac development to promote appropriate direction and remodeling of the looping heart tube.

Cardiac Septal Formation

Separation of oxygenated and deoxygenated blood is accomplished by septation of a common cardiac atrium and ventricle into right and left-sided chambers, which represents an evolutionary milestone for the transition from aquatic to terrestrial living. Accordingly, septal formation is complex and not completely understood (Webb et al., 1998; reviewed in Sadler, 1995). One model for dividing the lumen into two separate compartments may involve growth of a tissue mass that elongates until it reaches the opposite side of the lumen. Likewise, it may involve two growing primordia from opposite sides of the lumen that approach one another until they meet and fuse. In the developing heart, endocardial cushions, which give rise to the AVC and AVS, are thought to form the membranous non-muscular derived portions of the atrial and ventricular septae, as cushions swell to meet the

muscular portions derived from the myocardial wall of the atria or ventricle.

Alternatively, a septum may be formed if a portion of the chamber wall fails to grow, as surrounding tissue in the chamber continue to expand, resulting in a ridge that forms within an enlarging chamber and lumen. As the outgrowths expand, the two inner folds of the chamber wall may abut one another and fuse, leading to the formation of the septum. Although the formation of such a septum cannot completely divide the original lumen, it will leave a narrow communication between the two compartments that can be closed by a second proliferating tissue at the opposite side of the lumen, such as the endocardial cushions in the case of cardiac septae. Likely, contributions from components of each model of septal morphogenesis play a role for the formation of atrial and ventricular septae (Webb et al., 1998; reviewed in Sadler, 1995).

Several genes have been implicated to have a role in cardiac septal formation. From loss-of-function studies in mouse, several genes have been shown to be necessary for cardiac septation, and most, if not all, of these genes are also necessary for development of other cardiac and non-cardiac structures. For example, *Tbx1*, a member of the T-box gene family, lies within the DiGeorge region, the 22q11.2 locus, and is expressed in the pharyngeal arches which contribute cells to several anatomic structures, including the heart (Garg et al., 2001). Mice harboring targeted alleles of *Tbx1* display cardiac septal defects in addition to other elements of tetralogy of Fallot (Jerome et al., 2001; Merscher et al., 2001; Lindsay et

al., 2001) and non-cardiac components including cleft palate and hypoplasia of the thymus and parathyroid that is reminiscent of DiGeorge or velocardiofacial syndrome. *Jumonji (Jmj)*, a transcriptional repressor, was initially cloned in a gene trap screen to identify genes necessary for cardiac development (Toyoda et al., 2003; Kim et al., 2003). Homozygous *Jmj* mutants display a variety of defects that correlate with areas of *Jmj* expression, including ventricular septal defects, double-outlet right ventricle, and anomalies of the compact layer of the ventricular wall (Lee et al., 2000), in addition to proliferative defects of the megakaryocyte lineage (Kitajima et al., 2001).

Genetics of Congenital Heart Disease

Disruption of the developmental processes, described above, results in congenital heart defects (CHDs), the most common developmental anomaly and is the leading non-infectious cause of mortality in newborns (Hoffman, 1995). In humans, CHDs is commonly considered to be multifactorial in etiology, but in some rare cases, CHDs can be a monogenic disease of haploinsufficiency, often involving mild phenotypes with variable penetrance, which are evident at birth or later.

For these cases, linkage analysis of autosomal dominant syndromes has been informative for finding genetic culprits. First, mutations reveal the importance of uncharacterized or less characterized genes. For example, although pathogenetic mechanisms remain to be elucidated for supravalvular aortic stenosis

or Marfan's syndrome, studies of mutations in elastin and fibrillin, respectively, have helped to elucidate the significance of structural components of connective tissues in vascular biology and a better understanding of aortic disease (Curran et al., 1993; Lee et al., 1991; Maslen et al., 1991; Dietz et al., 1991).

Second, mutations causing autosomal dominant syndromes with cardiac defects have unveiled evidence for the role of unanticipated signaling pathways. For example, in *Drosophila*, Notch signaling was elegantly elucidated as a developmental pathway for establishing boundaries of cell fate (reviewed in Artavanis-Tsakonas et al., 1999). In the heart, a function for the Notch signaling pathway was first suggested with the identification of the Notch ligand, *Jagged-1*, as the causative gene for Alagille syndrome, a syndrome characterized by developmental defects of the heart, liver, eye and skeleton (Qi et al., 1997; Li et al., 1997a; Oda et al., 1997).

Finally, point mutations have revealed subtle but important roles for structural domains for many proteins, including transcription factors. For example, missense mutations in the T-box region of *TBX5* cause Holt-Oram syndrome, consisting of heart and limb anomalies (Basson et al., 1997; Li et al., 1997b). Patients with the G80R mutation within the T-box motif of TBX5 have more severe cardiac phenotypes, while patients with R237Q/W, another mutation within the T-box motif, have a more pronounced limb defect (Basson et al., 1999). Studies in vitro demonstrate that that G80R affects transcription of cardiac genes while R237Q does

not, suggesting functional deficits that correlate with phenotype (Hiroi et al., 2001). **Despite significant progress in this field, relatively little is known regarding the genes that cause disease in humans, and even less is understood about the possible mechanisms by which they may occur.**

Activation of Fetal Gene Program during Cardiac Hypertrophy

While CHDs are a leading cause of death in children, heart failure occurs more commonly in adults and is characterized by reactivation of many of the cardiac developmental gene programs described above. Heart failure, often secondary to valvular or congenital anomalies, cardiomyopathy, hypertension, or coronary artherosclerosis, is a leading cause of death in the Western world, and the incidence and prevalence continues to rise globally (Braunwald, 1994). Several adaptive mechanisms aid the heart if it faces an increased biomechanical stress in the context of pressure or volume overload, or the loss of myocardium. These modes include: increasing preload to the heart to increase the stretch of muscle fibers and consequent developed force for ventricular contraction; redistributing a lower cardiac output to sustain blood flow to vital organs such as the brain and the heart; regulating nonhumoral systems to preserve normal arterial pressure; and developing myocardial hypertrophy to normalize elevated ventricular wall tension (reviewed in Braunwald, 1994).

Although myocardial hypertrophy is considered an adaptive response for

maintaining cardiac output, prolonged hypertrophy may cause fibrosis and myofiber disarray, increasing the risk for dysrhythmia and arrhythmia, and can lead to sudden death or heart failure. Cardiac hypertrophy is characterized by increased myofiber and cardiomyocyte size, greater sarcomeric organization and the redeployment of components of the cardiac fetal gene program (reviewed in Hunter and Chien, 1999).

By using the rat neonatal cardiomyocyte as an in vitro system to complement transgenic mouse models, recent progress has been made in defining the key players that regulate cardiac gene expression directly by transcription factors or indirectly by kinases that affect transcription factors as targets (reviewed in Frey and Olson, 2003). For example, upon dephosphorylation by the phosphatase calcineurin, NFAT localizes to the nucleus where it cooperates with a cardiac enriched DNA-binding partner, Gata4, to activate cardiac gene expression (Molkentin et al., 1998). This pathway is opposed by the kinase GSK3, as phosphorylation of NFAT or Gata4 can lead to nuclear exclusion of both proteins (Beals et al., 1997; Morisco et al., 2001). In another example, MEF2C-dependent transactivation is inhibited by the recruitment of class 2 histone deacetylases (HDACs) (Lu et al., 2000a), but signals from CaMK in vitro or another HDAC kinase in vivo disrupts this interaction to promote shuttling of class 2 HDACs to the cytoplasm, allowing Mef2 to activate gene expression (McKinsey et al., 2000; Zhang et al., 2002).

Numerous transcriptional activators can regulate cardiac gene expression. Most interact with other transcription factors to cooperatively regulate downstream targets (reviewed in Akazawa and Komuro, 2003). A few transcriptional repressors have been identified, but the mechanisms by which many of them repress are less understood. Several ubiquitious signaling pathways have been implicated in the control of cardiac gene expression and cardiac hypertrophy, but the targets of these cascades are not well defined. Since modulation of myocardial growth may prevent many of the risks associated with cardiac hypertrophy, elucidation of signaling pathways that modulate the function of these transcriptional complexes provide the most attractive avenues for therapy, as small chemical compounds aimed at enzymes will likely be the most practical method for intervention (Sussman et al., 1998). **Therefore, an understanding of the underlying molecular mechanisms of cardiac gene expression may provide insights for the modulation of myocardial growth without affecting contractile function, as a possible approach to the prevention, correction or control of CHDs and heart failure.**

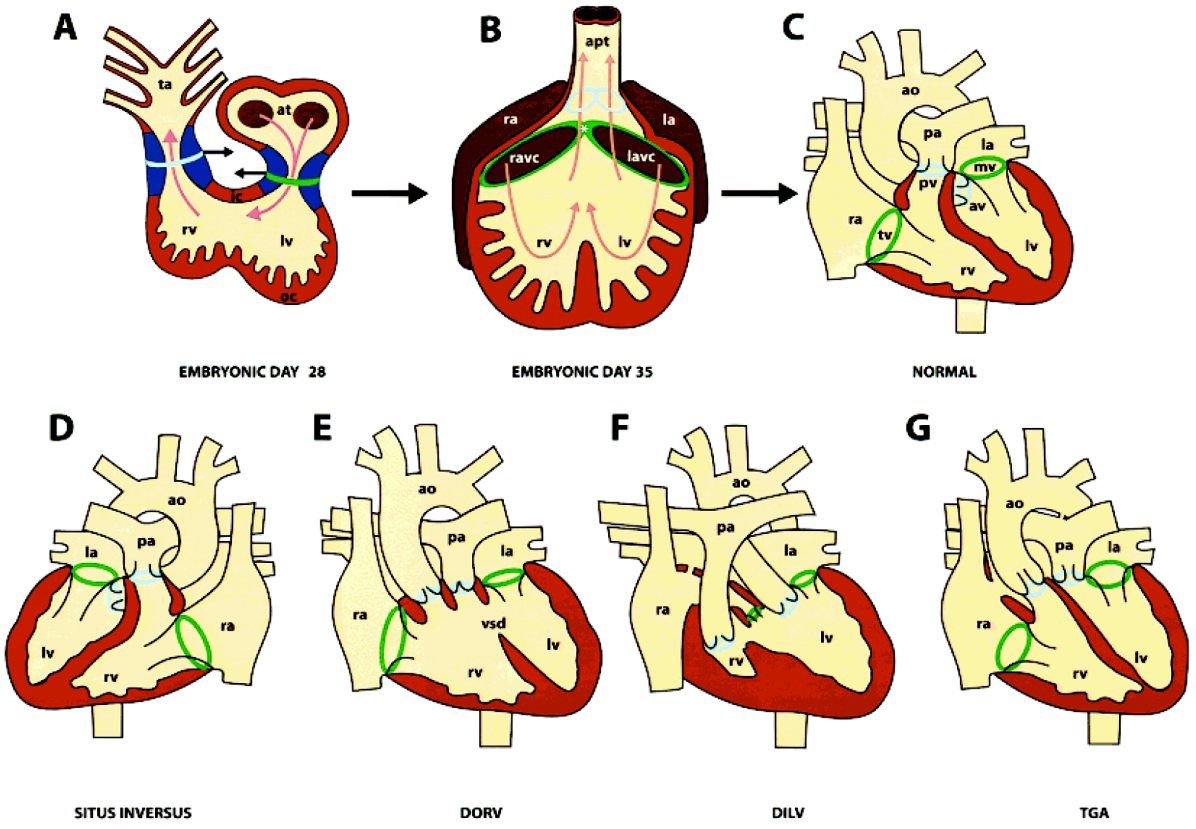


Figure 1. Normal and abnormal cardiac morphogenesis. (A) As the linear heart tube loops rightward upon inner curvature (ic) remodeling and outer curvature (oc) proliferation, the endocardial cushions (dark blue) of the inflow (green) and outflow (light blue) tracts become become adjacent to each other. Subsequently, the atrioventricular septum (AVS) shifts to the right, while the aortopulmonary trunk shifts to the left. ta, truncus arteriosus; at, atrium; rv, right ventricle; lv, left ventricle. (B) The inflow tract is divided into the right (ravc) and left (lavc) atrioventricular canal by the atrioventricular septum (asterisk). The outflow tract, known as the truncus arteriosus, becomes the aortopulmonary (apt) trunk upon septation. ra, right atrium; la, left atrium. Subsequently, the AVS shifts to the right. Similarly, the aortopulmonary trunk shifts to the left but also twists 180°. (C) Ultimately, the left atrium and right atrium are algned with the left ventricle and the right ventricle, respectively. The left ventricle and right ventricle become aligned with the aorta (ao) and the pulmonary artery (pa), respectively, after 180° rotation of the great vessels. mv, mitral valve; pv, pulmonary valve; tv, tricuspid valve. (D) If the determinants of the left-right axis are concordantly reversed, then a condition known as situs inversus results. (E) If the AVS fails to shift to the left, then a condition known as double-outlet right ventricle results, in which the right ventricle is aligned with both

the aorta and pulmonary artery. vsd, ventricular septal defect. (F) Likewise, if the AVS fails to shift to the right, then both atria communicate with the left ventricle in a condition known as double-inlet left ventricle (DILV). (G) Transposition of the great arteries (TGA) results if the aortopulmonary trunk fails to twist, resulting in communication of the right ventricle with the aorta and the left ventricle with the pulmonary artery (from Kathiriya and Srivastava, 2000).

METHODS

Plasmid Construction and Site-directed Mutagenesis

α-*MyHC*-luc (Molkentin et al., 1994), *ANF*-luc (Sprenkle et al., 1995), FLAG-TBX5 (Hiroi et al., 2001), myc-HRT1, myc-HRT2, myc-HRT3, myc-Hrt2(Δ33-37) (Nakagawa et al., 2000), myr-Akt/PKB (Akt*; Ballou et al., 2000), myr-Akt(T308A/S473A) (Kitamura et al., 1998), RSK2*/MAPKAPK (Poteet-Smith et al., 1999), CaMKIV* (Chatila et al., 1996), PKA (Mellon et al., 1989) were described previously by others; 6X GATA-luc was provided by S.R. Grant, and a mammalian expression construct of the dominant negative form of GSK3b (Xe et al., 1995) was provided by G. Crabtree. C. Lien generated *Nkx2.5*-luc (Lien et al., 1999) and GST-Gata4(177-322), and M. Murakami generated MBP-Hrt2. I. King constructed many of the Hrt2 mutations. For additional expression constructs, cassettes flanked by unique restriction sites were generated by PCR and cloned into pcDNA3.1-N-myc and –N-FLAG vectors (Invitrogen). Point mutations in Gata4 and TBX5 (Garg et al., 2003) or Hrt2 were introduced by generating two overlapping fragments by PCR and verified by sequencing.

Luciferase Assays

In 6 well plates, HeLa cells were transfected using Fugene 6 (Roche) with 300ng of reporter, 300ng of Gata4, 600ng of Gata4(mG295S), 300ng of Gata4(mE360del),

300ng of TBX5 or 600ng of Hrt2. Immunoblots were used to verify appropriate protein expression. Luciferase activity was measured 48 hours after transient transfection as previously described (Yamagishi et al., 2003). mG295S was consistently expressed at lower levels than wild type, necessitating transfection of two-fold greater plasmid to achieve similar protein levels. Neonatal rat ventricular cardiomyocytes were harvested as described below, and transfected with 150ng of reporter, 200ng of Gata4 and 400ng of TBX5, using Lipofectamine Plus reagent (Invitrogen).

Hrt2 mutations were titrated, such that protein expression of the mutations was comparable to protein levels of wild type Hrt2. 100ng of kinase reagents were used for the initial screen, and subsequent titration revealed that 5ng of Akt was sufficient for minimal changes of Gata4 transactivation and maximal responsiveness in the presence of Hr2 and Gata4. For data in part I, at least three independent experiments were performed in duplicate, with representative data shown. For data in part II, at least two independent experiments were performed in duplicate, with representative data shown. All experiments were accompanied by western analysis to verify protein expression.

Immunocytochemistry

48 hours after transient transfection with Fugene 6 (Roche), HeLa and COS1 cells grown on slides were fixed with 3.7% formaldehyde/PBS, permeabilized with 0.1%

TritonX-100/PBS, incubated with monoclonal anti-myc (Santa Cruz) and detected using anti-mouse conjugated-FITC antibody (Jackson Immunoresearch). Primary neonatal cardiomyocytes were plated on laminin-coated glass coverslips and fixed and stained with primary and secondary antibodies at 1:200 as above, with anti-actinin (EA-53. Sigma), anti-rat ANF (IHC 9103; Peninsula Laboratories, Inc), Cy3-conjugated anti-mouse or anti-rabbit IgG (Jackson Immunoresearch), and fluoroscein-conjugated anti-mouse or anti-rabbit IgG (Jackson Immunoreseach).

Electrophoretic Mobility Shift Assay

Annealed oligonucleotides (5'-TCGAGGTAATTAACTGATAATGGTGC-3') with a GGG overhang representing the GATA *cis* element upstream of *Hand2* (McFadden et al., 2000) were labeled with [^{32}P]dCTP using Klenow enzyme and incubated with 2λ of wild type and/or 2-8λ of mG295S Gata4 synthesized using the TNT Quick Coupled Transcription/Translation System (Promega) in binding buffer (20mM Hepes, pH7.9, 60mM Kcl, 1mM MgCl2, 0.5mM DTT and 10% glycerol) for 20 min at RT and separated on a 6% polyacrilamide gel (Charite et al, 1998). Radiolabeled reticulocyte lysate encoding myc-pcDNA was used to normalize the volume among samples. [^{35}S]-labeled protein products were verified by SDS-PAGE.

Co-immunoprecipitation and In vitro Binding Assays

COS1 cells were transiently transfected with Fugene 6 (Roche), harvested after 48

hours in lysis buffer (1XPBS, 1mM EDTA, pH8.0, 0.5% TritonX-100), incubated with polyclonal anti-myc antibody (Santa Cruz) and Protein A sepharose beads (Amersham Pharmacia), immunoblotted with monoclonal anti-myc (Santa Cruz) or anti-FLAG M2 (Sigma) antibodies and detected by the AP-conjugated CDP-Star system (Perkin Elmer). For overexpression of TBX5 point mutations and Hrt2 deletions, ^{35}S-labeled products were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega), incubated with COS1 lysates transfected with myc-Gata4 or myc-Nkx2-5, immunoprecipated with polyclonal anti-myc and visualized by autoradiography.

Cardiomyocyte preparation and adenoviral infection

Primary neonatal rat cardiomyocytes were prepared as described (Simpson and Savion, 1982) and plated in media containing Dulbecco Modified Eagle Medium (DMEM) and Medium 199 (M199) at a ratio of 4:1 (DMEM:M199), 10% horse serum, 5% fetal bovine seurm (FBS), 100μg/mL penicillin/streptomycin (P/S), and supplemented with 20mM L-glutamine, at a confluence of ~2-3x10^6 cells per 10cm culture dish. At least eighteen hours after plating, cells were washed with DMEM and infected with adenovirus in media containing 10% FBS for 2 hours, with manual rocking every 10-15 minutes. The cells were washed with DMEM and cultured in serum-free DMEM containing P/S for at least 24 hours, before stimulation with agonist. Cells were incubated in the presence of phenyleprhine (PE) (20μM,

Sigma), isoproteronol (Iso) (10μM; Sigma), or insulin-like growth factor-1 (IGF-1)(100ng/mL; Calbiochem) and harvested after 24 hours.

The recombinant adenovirus encoding myc-Hrt2 was prepared by Dr. R.D. Gerard, Department of Internal Medicine, by Cre/loxP-mediated recombination of a sub360 adenoviral cosmid (Aoki et al., 1999), which is an Ad5 derivative with a deletion in the E3 region, and a myc-Hrt2 expression plasmid. This construct was transfected into 911 cells (Fallaux et al., 1996) by lipofectamine 2000 (Invirogen). Primary lysates were used to reinfect 911 cells and viral plaques were obtained using the agar overlay method (Gerard and Meidell, 1995). Clonal populations of adenoviruses were amplified by reinfecting 911 cells and viral titer was determined.

Isolation and analysis of cardiomyocyte RNA and protein lysate

Cardiomyocytes was harvested using 1mL of TriZol (Invitrogen) per dish and total RNA was extracted and purified. RNA dot blot analyses were performed with 1ug of total RNA using radiolabeled oligonucleotides as probes. At least two independent experiments were performed for each condition. To harvest protein lysates from cardiomyocytes, .5mL of Laemmli buffer (Bio-Rad) per dish was used. 2% of cell lysate was utilized for western analysis.

PART I
MUTATIONS IN *GATA4* CAUSE CONGENITAL HEART DEFECTS AND REVEAL AN INTERACTION WITH TBX5

BACKGROUND

Genetics of Human Cardiac Septal Defects

Division of a common cardiac atrium and ventricle into right and left-sided chambers represents an essential evolutionary milestone in development of the four-chambered heart and is necessary for separation of oxygenated and deoxygenated blood. In humans, failure of atrial or ventricular septation accounts for nearly 50% of CHDs and requires open-heart surgery to restore normal circulation (Hoffman, 1995).

While cardiac septal defects (CSDs) are common, the precise molecular mechanisms for cardiac septal closure in humans remain to be elucidated. Mutations of the cell adhesion molecule, *CRELD1,* in humans result in atrioventricular septal defects (AVSD) in isolated AVSD and in the context of heterotaxy (Robinson et al., 2003). Mutations in *NKX2-5* have been identified in individuals with CSDs and conduction abnormalities, while individuals with Holt-Oram syndrome (HOS), characterized by CSDs, conduction abnormalities and limb anomalies, have point mutations in *TBX5* (Schott et al., 1999; Basson et al., 1997; Li et al., 1997b).

Nkx2-5 in cardiac development

Nkx2.5 is one of the earliest markers of the cardiac lineage. Identified as a potential homologue of *Drosophila tinman*, *Nkx2-5* is a homeodomain-containing transcriptional activator (Komuro and Izumo, 1993; Lints et al., 1993). The homeodomain of Nkx2-5 has a helix-turn-helix motif that binds to the specific consensus DNA sequence T(C/T)AAGTG (Chen and Schwartz, 1995) and can regulate several cardiac genes in vitro, including *ANF* and *Cxn40* (Durocher et al., 1996; Shiojima et al., 1999). Nkx2.5 cooperates with several co-partners, including Gata4, Mef2c, SRF, Tbx5, and dHAND (Shiojima et al., 1999; Durocher et al., 1997; Morin et al., 2000; Chen et al., 1996; Chen and Schwartz, 1996; Hiroi et al., 2001; Brueneau et al., 2001; Thattaliyath et al., 2002). Targeted disruption of *Nkx2-5* in mice causes embryonic lethality due to the arrested looping morphogenesis of the heart tube and growth retardation and reduction of several cardiac markers (Lyons et al., 1995; Tanaka et al., 1999). Following the discovery that human mutations of *NKX2-5* cause CSDs and conduction anamolies (Schott et al., 1999, Kasahara et al., 2000), mice harboring the targeted allele of *Nkx2.5* were reexamined and were found to have less pronounced but prevalent defects (Kasahara et al., 2001). These results are consistent with an important role for *Nkx2-5* in the transcriptional regulation of the cardiac gene program.

TBX5 and the T-box family of Transcription Factors

TBX5 belongs to the T-box family of transcription factors that is characterized by homology in a DNA-binding domain known as the T-box (Bollag et al., 1994; Chieffo et al., 1997). There are more than 20 known T-box family members in human, many of them conserved in different species, including *C. elegans, D. melanogaster*, zebrafish and mouse. Many of the well-studied members are expressed in a tissue-specific fashion during embryogenesis and adulthood (reviewed in Papaioannou and Silver, 1998; reviewed in Smith, 1999). The T-box family of transcription factors exhibit a sensitivity to gene dosage, as mice heterozygous for mutations in the *Brachyury* (T) gene have shortened or nonexistent tails (Kispert and Hermann, 1993), and heterozygous mutations of *TBX3* and *TBX5* have been shown to be the cause of the human ulnar-mammary syndrome and Holt–Oram syndromes (HOS), respectively (Bamshad et al., 1997; Basson et al., 1997; Li et al., 1997b). Targeted mutations in mice of *Tbx1,* one of the candidate genes in the del22q11 locus, display craniofacial and outflow tract anamolies reminiscent of the human DiGeorge syndrome (Lindsay et al., 2001; Jerome and Papaioannou, 2001; Merscher et al., 2001), while *Tbx3* or *Tbx5* display similar mouse phenotypes to their respective human diseases (Davenport et al., 2003; Bruneau et al., 2001), consistent with a monogenic etiology that is sufficient for these disease processes.

Tbx5 is necessary for appropriate cardiac development in humans, mice, fish

and frogs (Basson et al., 1997; Li et al., 1997b; Bruneau et al., 2001; Garrity et al, 2002; Ahn et al., 2002; Horb and Thompsen, 1999). Tbx5 regulates *Cxn40* and *ANF* in vivo and in vitro and can interact with Nkx2.5 (Bruneau et al, 2001; Hiroi et al., 2001). Overexpression of *TBX5* in chicken hearts by retrovirus results in decreased proliferation of embryonic cardiomyocytes (Hatcher et al., 2001) while mouse embryos that express *Tbx5* driven by a *beta-myosin heavy chain* promoter throughout the primitive heart tube display loss of ventricular-specific gene expression and retardation of ventricular chamber morphogenesis (Liberatore et al., 2000). In contrast, a stable cell line of pluripotent P19 cells expressing TBX5 induces the expression of cardiac markers (Hiroi et al., 2001). The discrepancy between in vivo and in vitro data may be attributed to sensitivity of the heart to *Tbx5* dosage, perhaps similar to *Tbx1*, in which too much or too little of *Tbx1* in the mouse causes similar defects (Merscher et al., 2001). Nonetheless, this evidence suggests a critical role for the control of gene expression by TBX5 in the heart.

Gata4 and the GATA family of Zinc Finger Transcription Factors

GATA4 belongs to a family of transcription factors that binds a consensus HGATAR DNA motif and contains two class IV zinc-finger domains (Evans and Felsenfeld, 1989; Tsai et al., 1989; Arceci et al., 1993). Gata4 and its orthologues regulate numerous downstream target genes to play a key role in cardiac development as well as myocardial differentiation and function (Durocher et al.,

1997), typically through combinatorial interactions with other cardiac-specific transcription factors, including Nkx2-5, SRF, NFAT3 and Fog2, as well as more ubiquitous factors such as the histone acetyltransferase, p300 (Durocher et al., 1997; Shiojima et al., 1999; Moore et al., 2001; Sepulveda et al., 2002; Molkentin et al., 1998; Tevosian et al., 1999; Lu et al., 1999; Svensson et al., 1999; Dai and Markham, 2001).

Gata1,-2 and *-3* are expressed predominantly in hematopoietic cells and heterozygous mutations of *GATA1* and *GATA3* cause human blood disorders and organ malformations, respectively (Nichols et al., 2000; Van Esch et al., 2000). In contrast, *Gata4, -5,* and *-6* are expressed in the developing heart and in several endodermal lineages, but have not been implicated in human disease (Arceci et al., 1993; reviewed in Molkentin, 2000). However, null mutations in the Drosophila *Gata4* orthologue, *pannier* or zebrafish *Gata5* result in early defects in cardiogenesis (Gajewski et al, 1999; Reiter et al., 1999). Similarly, homozygous disruption of *Gata4* in mice results in failure of midline fusion of the heart tube resulting in early embryonic lethality (Molkentin et al, 1997; Kuo et al., 1997). Although mice heterozygous for *Gata4* do not have obvious cardiac anomalies, further reduction in Gata4 dosage from a hypomorphic *Gata4* allele causes cardiac septal and other congenital heart defects (W. Pu and S. Izumo, personal communication).

Interestingly, mutations of GATA co-factors display defects in endocardial cushion formation, including cardiac septal defects and valve anomalies. For

example, mice harboring targeted mutant allele of the GATA co-activator NFATc1 display cardiac septal defects as well as thickened semilunar valves (Ranger et al., 1998; de la Pompa et al., 1998). Targeted disruption of the GATA co-repressor, Fog-2, results in cardiac septal defects, in addition to valvular atresia and thin myocardium (Svensson et al., 2000; Tevosian et al., 2000), and a point mutation of *Gata4* that is predicted to ablate the interaction with Fog-2 phenocopies the ventricular septal defect and valve defects present in *Fog-2* null animals (Crispino et al., 2001). In the second chapter of this thesis work, we define an additional GATA co-repressor to be Hrt2. Targeted deletion of *Hrt2* in mice result in ventricular septal defects and valvular defects among other cardiac anomalies (Sakata et al., 2002; Gessler et al, 2002; Donovan et al., 2002). As stated previously, linkage analyses have revealed that NKX2.5 and TBX5 are necessary for appropriate cardiac septal formation in humans (Schott et al., 1999; Basson et al., 1997; Li et al., 1997b). Others have shown that Nkx2.5 is a GATA co-factor and this thesis work presents evidence to suggest that TBX5 is also a GATA co-partner (Garg et al., 2003). Taken together, such evidence would predict that cardiac septal formation and valve formation are sensitive to GATA dosage.

RESULTS

Mutations in *GATA4* cause Congenital Heart Defects

Drs. Reenu Eapen and Vidu Garg identified a large kindred spanning five generations in which sixteen individuals had CHDs (Figure 1a). After reviewing clinical evaluations for all available family members, the phenotype demonstrated an autosomal dominant pattern of inheritance. All fifteen affected family members had atrial septal defects, of which three were undiagnosed prior to the study. Eight individuals had additional forms of CHDs, including ventricular septal defects, atrioventricular septal defects, pulmonary valve thickening, or insufficiency of cardiac valves (Figure 1b, c). Unlike the familial cases of CSDs associated with mutations of *NKX2-5* and *TBX5*, neither the cardiac conduction system nor other organs were affected in this kindred, suggesting that the CHDs were isolated and not associated with multi-organ syndromic disease.

Direct sequencing of *NKX2-5* and *TBX5* in this family by others failed to reveal mutations, suggesting an alternative genetic etiology. To identify the general chromosomal region that was linked to disease in this family, Vidu Garg, Assistant Professor of Pediatrics, in the lab, performed a genome-wide scan and his analysis revealed linkage of the CHD phenotype to a single locus on chromosome 8p22-23 (LOD score=5.7, θ=0) between D8S264 and D8S1827, spanning approximately 30 cM (~12.7 Mb). Examination of genes in this interval revealed the presence of *GATA4*, which encodes a zinc-finger transcription factor essential for cardiogenesis

in flies, fish and mice (Gajewski et al., 1999; Reiter et al., 1999; Molkentin et al. 1997; Kuo et al., 1997). Direct sequencing of *GATA4* in an affected family member identified a G⇒A transition of nucleotide 886 (Figure 1d) that predicted a glycine to serine substitution at codon 296 (hG296S) (Figure 2b). All affected individuals that were clinically evaluated had the hG296S mutation, suggesting complete penetrance of the disease phenotype (Figure 1d). The mutant allele was not detected in unaffected family members nor in 3000 unrelated individuals of diverse ethnicity, making it unlikely that hG296S represented a rare polymorphism. In the proband, Dr. Garg sequenced one hundred additional regulatory genes essential for, or expressed during, cardiac development but failed to identify other linked mutations, consistent with a monogenic etiology (V.G. and D.S., unpublished data).

By direct sequencing of *GATA4*, Dr. Garg, in collaboration with Rumiko Matsuoka of Tokyo Women's University, identified a second family with autosomal dominant transmission of atrial septal defects in which a mutation of *GATA4* (hE359del) was found in all available affected members spanning four generations (Figure 1e, f). Similar to family A, neither the cardiac conduction system nor other organs were affected in this kindred. The hE359del mutation in this pedigree resulted in a frame shift past amino acid 359, thus severely altering the encoded GATA4 protein (Figure 1g). A premature stop codon is predicted by the frame shift and may result in a truncated protein or nonsense-mediated decay of the transcript (Frischmeyer et al., 2002). This nucleotide deletion was not found in unaffected

family members or in 300 other individuals. The segregation of *GATA4* mutations with cardiac septal defects in two large unrelated families provides strong evidence that mutations in *GATA4* are the underlying cause of a subset of familial, non-syndromic cardiac septal defects. Consistent with this, deletion of the terminal end of chromosome 8p that contains *GATA4*, among many other genes, is characterized by cardiac septal defects (Pehlivan et al., 1999).

GATA4* mutations display functional deficits *in vitro

The hG296S mutation affects a residue that is highly conserved across species, including yeast, and among other GATA family members. The glycine residue lies immediately adjacent to the nuclear localization signal (NLS) and the carboxy-terminal zinc finger, while the hE359del mutation results in truncation of the last forty amino acids (Figure 2a,b). To determine if the GATA4 mutation altered wild type GATA4 function, I generated both mutations with the help of Drs. V. Garg and I. King, in the highly conserved mouse orthologue (mG295S or mE360del).

To determine if the Gata4 mutations affected the ablility of Gata4 to activate transcription of downstream genes, I tested their abilities to activate transcription of downstream genes in vitro, utilizing several GATA-dependent cardiac enhancers upstream of a luciferase reporter. When equivalent amounts of protein were expressed, mG295S displayed less transcriptional activation of the *alpha myosin heavy chain* (*α-MyHC*) (Molkentin et al., 1999), *atrial natriuretic factor* (*ANF*)

(Sprenkle et al., 1995) and *Nkx2.5* (Lien et al., 1999), compared to wild type, suggesting mildly reduced activity in this overexpression system (Figure 2c and data not shown).

In contrast, the truncated protein generated from recombinant mE360del was unable to activate transcription of either the α-*MyHC* or *ANF* reporter (Figure 2c). It will be interesting to determine if mE60del can interfere with wild-type function, since many domains are intact. While it cannot be determined if transcripts encoding hE359del undergo nonsense-mediated decay in vivo, the inability of mE360del to activate transcription suggests that GATA4 is likely haploinsufficient. The generation of mice harboring a mutant allele encoding m360del will be necessary to determine if nonsense-mediated decay occurs in vivo.

Because mG295S functioned as a hypomorph, I investigated the mechanisms through which this mutation affected Gata4 function. Although mG295S is near the NLS, Dr. King and I observed that the protein localized to the nucleus in HeLa and COS1 cells, suggesting that the NLS was not disrupted (Figure 2d, data not shown).

mG295S is also immediately adjacent to the carboxy-terminal zinc finger, which is essential for DNA-binding and interaction with co-factors (Morrisey et al. 1997) (Figure 2a,b). In electromobility shift assays (EMSA) to test DNA-binding affinity, wild type Gata4 efficiently retarded gel migration of a ^{32}P-labeled GATA *cis* element corresponding to the *Hand2* enhancer (McFadden et al., 2000). In contrast, equivalent amounts of mG295S displayed limited affinity for the GATA element

(Figure 2e). With two- or four-fold excess of mG295S, we observed only weak DNA-binding. To test if the mG295S mutant affected wild-type Gata4 binding to DNA, wild type Gata4 was co-expressed in the presence of increasing amounts of mG295S, no alteration of wild type DNA-binding affinity was detected (Figure 2e). Together, these data suggest that the impaired DNA-binding of Gata4 mG295S may contribute to the reduced transcriptional activity of the mutant, although forced overexpression of the hypomorphic protein in tissue culture may compensate for the decreased DNA-binding affinity.

Gata4(G295S) fails to interact with TBX5

The zinc-finger domains of Gata4 also mediate numerous protein-protein interactions that often dictate DNA-binding specificity and therefore, subsets of target gene activation. Hence, I tested if mG295S affected Gata4's ability to interact with other transcriptional regulators, including two implicated in human cardiac septal formation, NKX2-5 and TBX5. By immunoprecipitation, m295S was able to interact with Nkx2-5 via the carboxy-terminal zinc-finger, similar to wild type Gata4 (Durocher et al., 1997), suggesting that this motif was intact (Figure 3a). In addition, I discovered a novel interaction between Gata4 and TBX5, as both wild type proteins could immunoprecipitate with one another (Figure 3b). However, mG295S could not interact with wild type TBX5, suggesting specific disruption of the Gata4-TBX5 interaction by the Gata4 mutation (Figure 3b).

To determine whether Gata4 cooperates with TBX5 to activate transcription, I co-transfected the *ANF* reporter with TBX5 and Gata4 in HeLa cells, 10T1/2 fibroblasts and primary cardiomyocytes. Similar to Tbx5-Nkx2-5 interactions (Hiroi et al., 2001; Bruneau et al., 2001), co-expression of TBX5 and Gata4 resulted in cooperative activation of the reporter (Figure 3c, data not shown). Consistent with this observation, when I co-expressed Gata4 and TBX5, I was unable to detect an increase in transactivation of a luciferase reporter downstream of six tandem GATA sites (data not shown). I was also unable to detect a tertiary complex of TBX5 and Gata4 with the GATA *cis* element by EMSA (data not shown), suggesting that additive transactivation of the complex may require DNA-binding of both Gata4 and TBX5. Because of the additive nature for transactivation by Gata4 and TBX5, these in vitro assays were not conclusive in determining the effect of the mG295S Gata4 mutant with TBX5.

Human mutations of TBX5 affect binding to Gata4 and Nkx2-5

The similarity between cardiac septal defects observed in the setting of human *GATA4* mutations and human *TBX5* mutations is consistent with the Gata4-TBX5 complex described here. Human *NKX2-5* mutations also cause similar septal defects and NKX2-5 can physically interact with TBX5 (Hiroi et al., 2001; Bruneau et al., 2001). I therefore asked if reported human *TBX5* mutations affect the ability of TBX5 to interact with GATA4 or NKX2-5. Six unique *TBX5* missense mutations from

families that display HOS (Basson et al., 1999; Cross et al., 2000; Yang et al., 2000) (Figure 4a) were examined for their ability to interact with wild-type Gata4 or Nkx2-5. Since the point mutations of TBX5 were expressed at various levels in COS1 cells, I utilized an in vitro binding assay, in which COS1 lysates overexpressing myc-Gata4 or myc-Nkx2.5 were combined with radiolabeled TNT products expressing protein encoded by each TBX5 point mutation construct. Subsequest immunoprecipitation using anti-myc antibody was performed and output lysates were run on SDS-PAGE and exposed to autoradiography. In this assay, all TBX5 mutants were able to bind to Gata4 or Nkx2-5, except for the G80R and R237W mutants (Figure 4b). In contrast, G80R or R237W were able to interact with several other cardiac transcription factors, including Hrt2 (data not shown), suggesting specific disruption of TBX5 - Gata4/Nkx2-5 complexes. The reciprocal evidence that human *GATA4* and *TBX5* mutations affect interaction with one another in the setting of similar CHDs provides support that GATA4 and TBX5 may cooperate during cardiogenesis.

DISCUSSION

By complementing the human genetics performed by others, my biochemical analyses helped to reveal a previously unrecognized genetic etiology for CHDs and potential mechanisms through which certain cardiac defects may occur. The mutations in *GATA4* suggest that haploinsufficiency of *GATA4* in humans can result in the most common types of cardiac malformations and that GATA4 is essential for functional separation of the four cardiac chambers. In particular, the G296S mutation of GATA4 demonstrated not only an effect on DNA-binding affinity and transactivation of downstream targets, but also revealed an interaction between GATA4 and TBX5 that is disrupted in the setting of CHD. The similar effect of TBX5 mutations on the interaction with GATA4 and NKX2-5 raises the possibility that TBX5, NKX2-5 and GATA4 function in a complex to regulate of a subset of genes required for cardiac septal formation. While haploinsufficiency of *TBX5* has been linked to cardiac septal malformation in HOS, the disruption of a GATA4-TBX5 complex by some *TBX5* point mutations provides a potential mechanistic understanding of how disruption of combinatorial interactions of transcription factors can lead to specific birth defects.

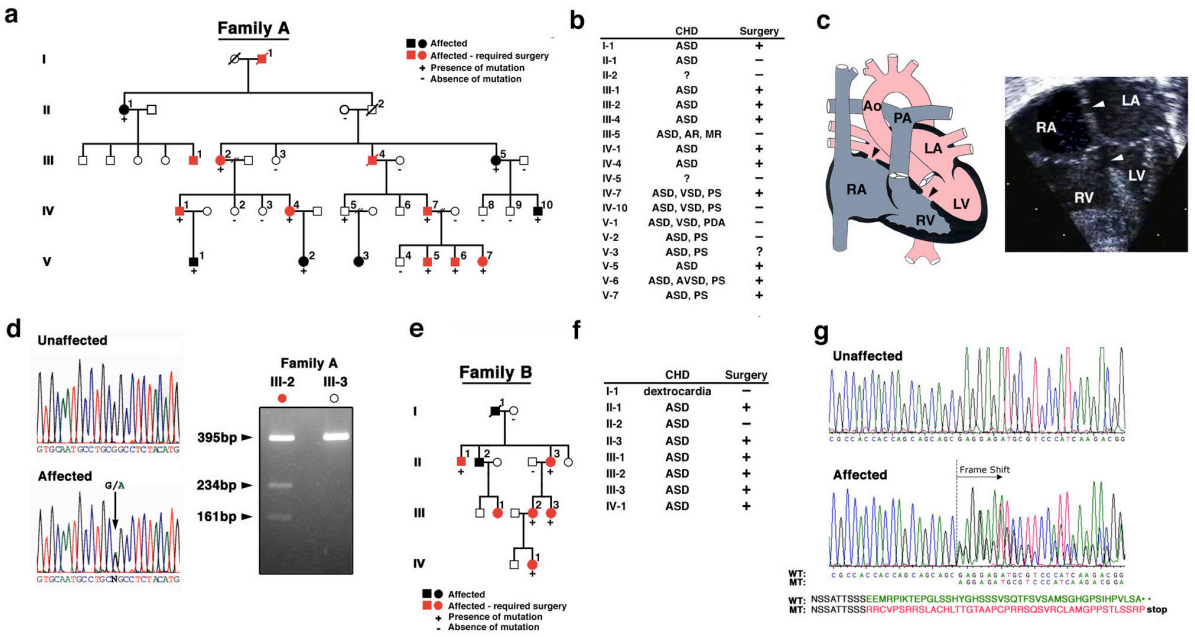
Within a single organ, it is notable that pleiotropic defects involving cardiac septal formation and valvular development can be observed in individuals with a shared mutation, as described here, although many of the defects can be derived from a common embryologic origin, the endocardial cushions. This phenomenon

has also been described for *NKX2-5* mutations and suggests a role for modifying genetic, environmental or stochastic events even for monogenic causes of cardiac defects. Since CSDs are typically isolated and occur in the absence of conduction anomalies, the *GATA4* mutations described in these families without other anomalies may prove to be relevant to a broader range of patients with such defects. We have already found *GATA4* mutations in other cases of familial CSD and possibly in sporadic cases.

It is interesting that some patients with cardiac septal defects have more rapid or severe cardiac dysfunction than others, requiring earlier or more common surgical repair. Because GATA4 is a central factor in transcriptional regulation of cardiac hypertrophy and heart failure, it will be interesting to determine whether mutations of GATA4 as a genetic etiology of CHDs segregate with an increased incidence of progressive cardiac dysfunction. Broad screening for *GATA4* mutations in humans with heart disease may provide new avenues for understanding the development of CHDs, leading to future therapeutic or preventive interventions for a leading cause of mortality of children.

In the future, the generation of mice harboring the human *Gata4* mutant alleles may provide important genetic tools for understanding the cellular underpinnings of cardiac septal defects, while the generation of compound heterozygote mice harboring mutant alleles of *Gata4* and *Tbx5* will be useful for identifying a potential genetic interaction between *Gata4* and *Tbx5*. In addition, the

functional analysis of additional mutations in *GATA4* may provide inroads for the study of transcriptional mechanisms relevant to CHDs.



	CHD	Surgery
I-1	ASD	+
II-1	ASD	–
II-2	?	–
III-1	ASD	+
III-2	ASD	+
III-4	ASD	+
III-5	ASD, AR, MR	–
IV-1	ASD	+
IV-4	ASD	+
IV-5	?	–
IV-7	ASD, VSD, PS	+
IV-10	ASD, VSD, PS	–
V-1	ASD, VSD, PDA	–
V-2	ASD, PS	–
V-3	ASD, PS	?
V-5	ASD	+
V-6	ASD, AVSD, PS	+
V-7	ASD, PS	+

	CHD	Surgery
I-1	dextrocardia	–
II-1	ASD	+
II-2	ASD	–
II-3	ASD	+
III-1	ASD	+
III-2	ASD	+
III-3	ASD	+
IV-1	ASD	+

Figure 1. *GATA4* mutations segregate with familial cardiac septal defects. (a) Kindred with five generations, indicated in Roman numerals, affected by congenital heart defects (shaded circles or squares). Subjects shaded in red required surgical repair of the cardiac defect. + or - indicates presence or absence of G296S mutation, respectively. Mutation analysis was not performed on expired family members and III-1 and V-3, secondary to incarceration and inability to contact, respectively. Echocardiography or operative data was available for all subjects except for I-1, II-2, and IV-5 and are summarized in (b). ASD, atrial septal defect; VSD, ventricular septal defect; PS, pulmonary stenosis; PDA, patent ductus arteriosus; AVSD, atrioventricular septal defect; AR, aortic regurgitation; MR, mitral regurgitation. (c) Echocardiogram and schematic interpretation of ASD and VSD in representative family member, indicated by arrowheads. (d) Genome-wide scan revealed linkage to 8p23 in all affected subjects (LOD=5.7). G to A substitution of nucleotide 886 in the *GATA4* gene was present in all affected, but not unaffected, members of the family tested and was not detected in 3000 controls. The mutation introduced a new PstI restriction enzyme site in exon 3 resulting in appearance of 234bp and 161bp fragments (III-2) upon PstI digestion of the 395bp wild type *GATA4* exon 3 (III-3). (e, f) Second pedigree with eight subjects across four generations affected by atrial septal defects (II-1, 2, 3, III-1,2,3, IV-1) or dextrocardia (I-1, died at 78 years of age). Those requiring surgical repair are indicated in red. (g)

Six (I-1, II-1,2, III-1,2, IV-1) had a deletion of nucleotide 1075 in exon of 5 of *GATA4* that was not present in unaffected family members or in 100 race-matched controls. This deletion resulted in a frameshift mutation that altered the GATA4 amino acid sequence after amino acid 359 and resulted in a premature stop codon. Two (II-3, III-3) affected members were not available for the mutation study (from Garg et al., 2003).

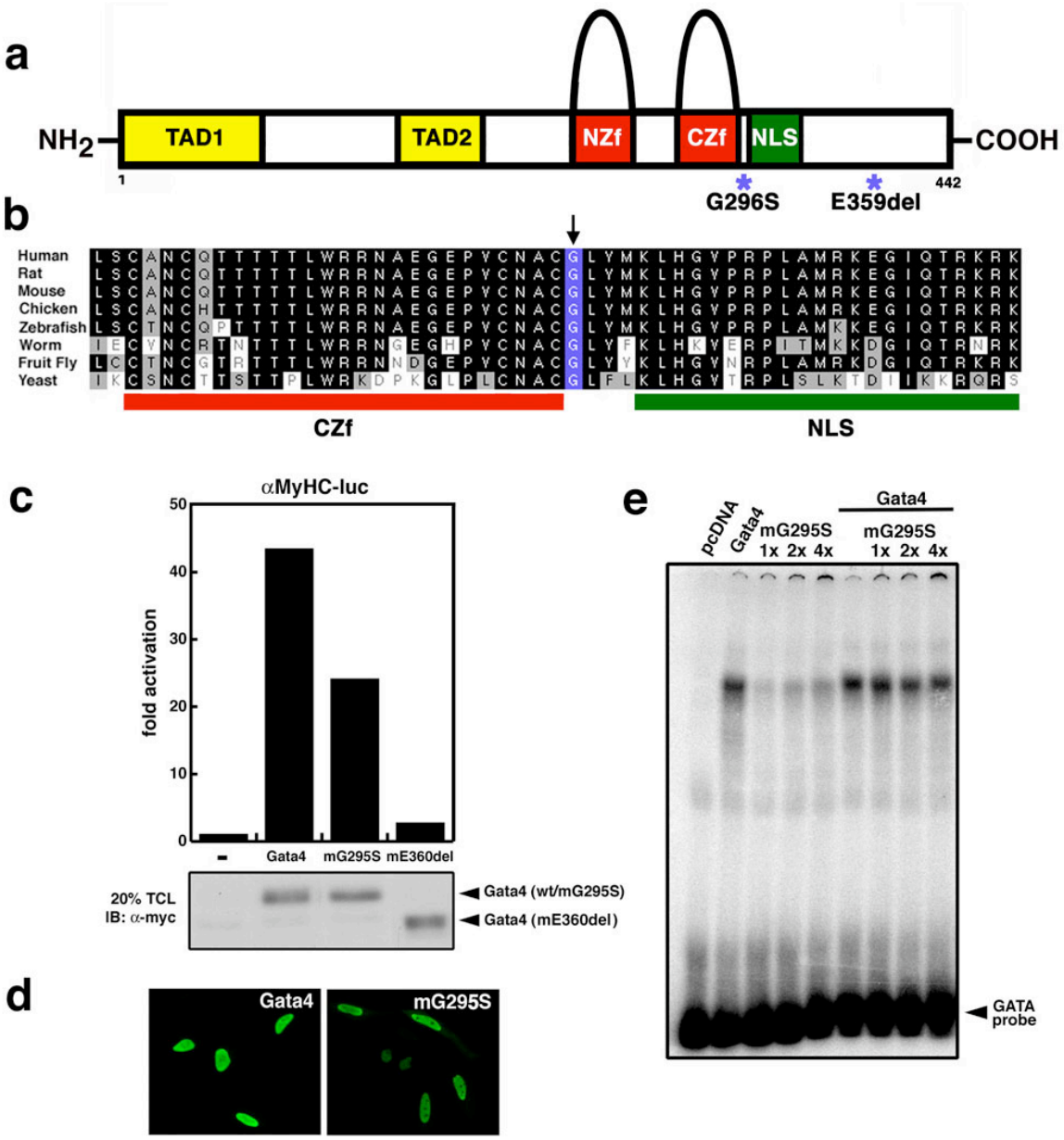


Figure 2. Functional deficits associated with Gata4 mG295S mutation. (a) Schematic of GATA4 protein domains indicates transactivation domains (TAD), N-terminal zinc-finger (NZf), C-terminal zinc-finger (CZf), and nuclear localization signal (NLS). Location of G296S or E359del mutations is indicated. (b) Cross-species alignment of amino acids in region of G296S mutation (highlighted in blue). The carboxy-terminal zinc-finger is indicated in orange; NLS is indicated in green. (c) Wild-type or mutant Gata4 activation of *ANF*- or α*–MyHC*-luciferase reporters in HeLa cells. Values are an average of fold-activation above baseline of duplicates

from a representative of three independent experiments. TCL, total cell lysate. (d) Subcellular localization of myc-tagged wild type or G295S Gata4 overexpressed in HeLa cells was detected in the nucleus by indirect immunoflourescence, represented in green. (e) Electromobility shift assay revealed limited ability of mG295S Gata4 to retard migration of ^{32}P-labeled GATA *cis* element. Increasing amounts of mutant protein, up to four-fold greater than wild type, showed some residual DNA-binding ability. Excess mutant protein was unable to inhibit wild type DNA-binding (from Garg et al. 2003).

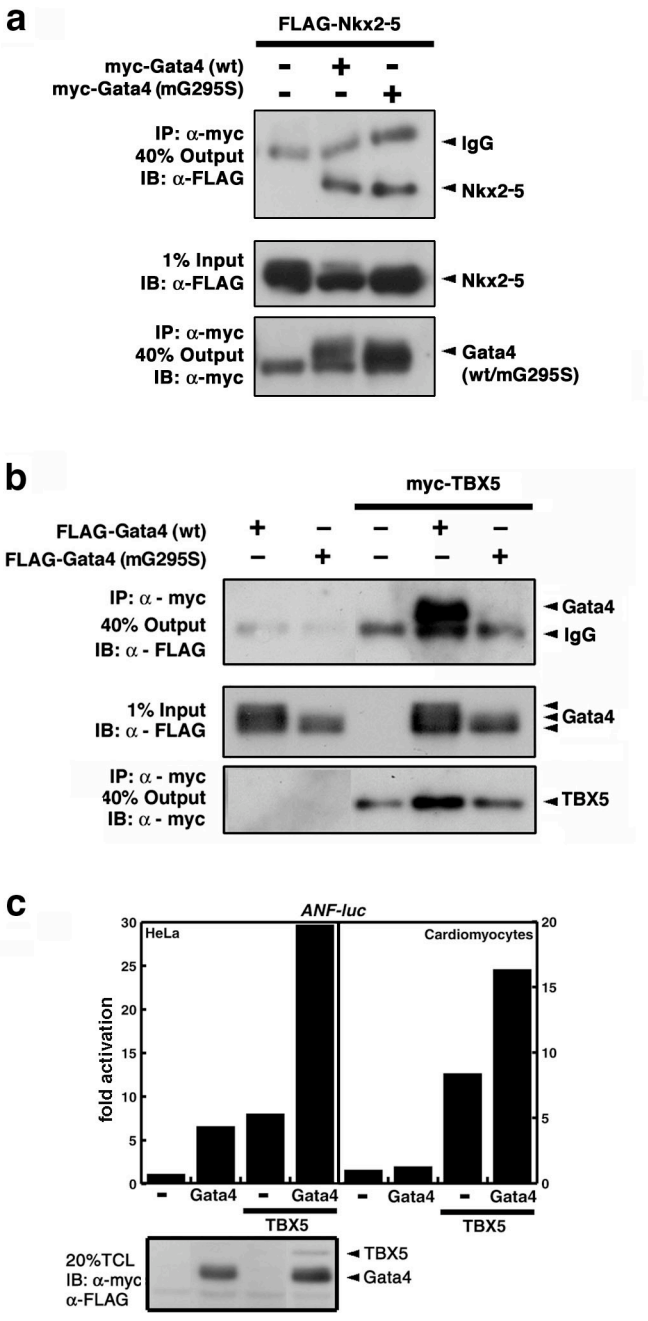


Figure 3. Gata4 interaction with TBX5 is specifically disrupted by Gata4 G295S mutation. (a) Immunoprecipitation (IP) of myc-tagged wild type or mutant Gata4 with an a-myc antibody revealed association of both with Nkx2-5 by a-FLAG immunoblot (IB). (b) IP of myc-tagged TBX5 with an a-myc antibody revealed an association of wild type FLAG-Gata4 with TBX5. The mG295S form of Gata4 disrupted interaction with TBX5. Protein expression for input and output are shown. (c) Luciferase activity directed by the *ANF* promoter in the presence of Gata4 and TBX5 in HeLa or cardiomyocytes. Protein levels of Gata4 and TBX5 are indicated. Activity is indicated as fold change over baseline (from Garg et al., 2003).

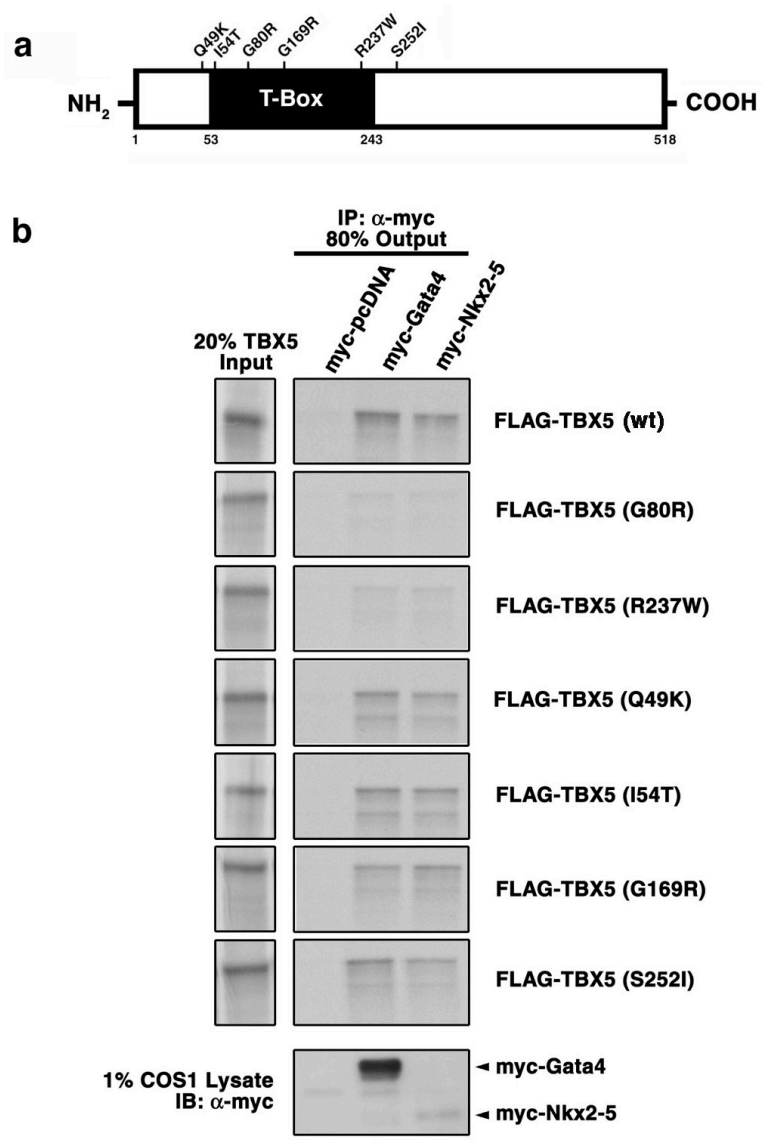


Figure 4. Interaction of human TBX5 mutants with Gata4 and Nkx2-5. (a) Schematic of previously identified mutations of TBX5 in humans with cardiac septal and limb defects (Holt-Oram Syndrome). Location of T-box is indicated. (b) All ^{35}S-labeled TBX5 mutants bound to myc-Gata4 and myc-Nkx2-5 similar to wild type FLAG-TBX5, except for the G80R and R237W mutants, when immunoprecipitated with an α-myc antibody. Input of ^{35}S-labeled TBX5 proteins and overexpression of myc-Gata4 and myc-Nkx2-5 in cell lysates are shown (from Garg et al., 2003).

PART II
AKT-SENSITIVE INHIBITION OF GATA-DEPENDENT CARDIAC GENE EXPRESSION BY HAIRY-RELATED TRANSCRIPTION FACTORS

BACKGROUND

Activation of cardiac gene program by growth factors

Several autocrine and paracrine growth stimuli may contribute to cardiomyocyte growth, known as eutrophy during development and hypertrophy in disease. They can include the involvement of receptor-tyrosine kinase cascades activated by insulin-like growth factor-1 (IGF-1) or platelet-derived growth factor (PDGF), and G-protein coupled receptor signaling, including the β- and α-adrenergic (βAR, αAR) pathways as well as angiotensin II (AngII) and endothelin-1 (ET-1) (reviewed in Frey and Olson, 2003). These extracellular hypertrophic signals can traverse the cell via second messengers, many of which, if not all, are calcium-dependent regulators, and likely converge on a set transcription factors to modulate gene expression in the nucleus (reviewed in Akazawa and Komuro, 2003). Yet, relatively little molecular detail is known regarding how these effectors might function at the terminal steps to influence transcriptional regulators, in order to reprogram cardiac gene expression.

PI3K-Akt pathway can mediate hypertrophic stimuli

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a critical regulator of cell size and cell survival in many tissues (reviewed in Brazil and Hemmings, 2001; reviewed in Datta et al., 1999). Akt is a principle target of the phosphoinositide 3-kinase (PI3K) pathway and can mediate the signaling of several hypertrophic stimuli in cardiomyoctyes, including IGF-1, the βAR agonist Isoproteronal (Iso) (Morisco et al., 2000), and less so by ET-1 (reviewed in Sugden, 2001). Similar to overexpression of a constitutively active form of PI3K in the heart (Shioi et al, 2000a), misexpression of a constitutively active form of Akt by a cardiac-specific enhancer in mice resulted in characteristics of cardiac hypertrophy, including increases in cardiomyocyte size, concentric left-ventricular (LV) hypertrophy (Condorelli et al., 2002). Misexpression of a kinase dead form of Akt did not affect chamber or myofiber size, but blocked induction of hypertrophy by overexpression of PI3K (Shioi et al., 2002).

Transcriptional regulation of β-adrenergic induction of cardiac hypertrophy by Akt may be mediated, in part, by phosphorylation and inhibition of its target, glycogen synthase 3-kinase (GSK3) (Morisco et al, 2000). GSK3 phosphorylation of cardiac transcriptional activators, Gata4 and NFAT3, results in nuclear export of Gata4 and NFAT3 in cardiomyocytes and likely reduces cardiac gene expression (Morisco et al., 2001; Antos et al., 2002). Therefore, Akt may relieve GSK3 inhibition as one potential mechanism for reprogramming cardiac gene expression.

Gata4 induces cardiac gene expression

Gata4 has been implicated as a key mediator, as β- and α-adrenergic pathways, via different second messengers, converge to regulate Gata4 transactivation (Morisco, 2001; Liang et al., 2000b; Liang et al., 2001; Morimoto et al., 2000, 2001). Gata4 or –6 are sufficient and necessary for induction of cardiomyocyte hypertrophy in rat neonatal cadiomyocyte primary cultures and overexpression of Gata4 in mice in the adult heart demonstrated a progressive increase in heart to body weight ratio and histologic features of cardiomyopathy, in addition to activation of hypertrophy-associated gene expression (Ling et al., 2001a). Evidence also suggests that GATA factors may be effectors of the MAPK/ERK, RhoA and GSK3 signaling pathways (Liang et al., 2001b; Yanazume et al., 2002; Charron et al., 2001; Morisco et al., 2001). In addition, in vitro transfection studies in non-cardiac cells has established Gata4 as an activator of several cardiac promoters, including *atrial natriuretic factor (ANF)* and *alpha myosin heavy chain (α–MyHC),* via interactions with several transcriptional co-repressors, including Fog2, as well as DNA binding co-activators, such as NFAT3, a target of a calcineurin-dependent pathway for cardiac hypertrophy (reviewed in Molkentin, 1999).

***Hrt* genes as potential modulators of fetal cardiac gene program**

Members of the hairy-related transcription factor family (Hrt; also known as Hey, Chf, grl, Hesr, Herp by other groups) contain a conserved bHLH motif, which mediates DNA and protein interactions, an orange domain and a C-terminal motif (Nakagawa et al., 1999; Leimester et al., 1999; Chin et al., 2000; Zhong et al., 2000; Kokubo et al., 1999; Iso et al. 2001a). *Hrt* genes were initially identified as intriguing components of the cardiovascular gene program, as *Hrt1* is restricted to the atria, *Hrt2* to the ventricles while all three members are expressed in the developing vasculature and the adult heart. Studies in zebrafish revealed that a mutant of the orthologous Hrt gene, *gridlock*, displayed coarctation of the aorta, while loss-of-function assays using morpholinos and gain-of-function experiments using RNA injection demonstrated that *gridlock* expression favors the development of arteries over veins (Zhong et al., 2000; Zhong et al. 2001). In mice, targeted disruption of *Hrt2* revealed a spectrum of congenital cardiac anomalies and cardiomegaly (Gessler et al., 2002; Donovan et al., 2002; Sakata et al., 2002).

Studies have revealed that members of the Hrt protein family can function as transcriptional repressors in vitro. Hrt2 can repress Notch-dependent transactivation by a mechanism of repression that is DNA-independent (Nakagawa et al., 1999, PNAS) and likely to be via protein-protein interactions between Hrt2 and the obligate DNA-binding partner of Notch, suppressor of hairless (Su(H)) (King et al., unpublished). Hrt2 can recruit other co-repressors, including class 1 and class 3 HDACs as well as NCoR (Iso et al., 2001b; Takata and Ishikawa, 2003). Hrt

proteins can also inhibit skeletal myogenesis, by inhibiting the formation of the E12-MyoD heterodimer, by directly interacting with MyoD (Chin et al., 2001). Although *Hrt-1* and *–2* expression are enriched in the heart, it remains unknown if and how Hrt proteins can repress cardiac gene expression.

RESULTS

Hrt2 inhibits markers of cardiomyocyte hypertrophy

Since members of the *Hrt* gene family are important during cardiovascular development and in the adult heart, I asked if the Hrt proteins could alter the expression of the cardiac fetal gene program in the setting of cardiomyocyte hypertrophy. We utilized a well-established method for assaying cardiac hypertrophy in vitro. In the presence of growth stimuli, for example, the α-adrenergic agonist phenylephrine (PE), neonatal rat cardomyocytes increase in cell size, exhibit a higher degree of sarcomeric organization, as well as the induction of several fetal markers, including *ANF*.

I infected cardiomyocytes with adenoviruses overexpressing Hrt2 (AdHrt2) or control (Adcont) with multiplicity of infection (MOI) at 3, 10, 30 and 100 pfu/cell, and incubated them with vehicle or the agonist, PE. Cells were harvested at 18, 24 and 36 hours. I examined for alterations in morphological changes characteristic of cadiomyocyte hypertrophy, such as increases in sarcomeric organization, using an antibody to a component of the sarcomere, α-actinin (Figure 1A). With vehicle, cell populations infected with or without AdHrt2 were nearly indistinguishable, suggesting AdHrt2 at an moi of 30 pfu/cell, in part, was not apparently toxic to the cells by fluorescence microscopy. With PE treatment, uninfected cells or those infected with Adcontrol underwent dramatic organization of their sarcomeres, reminiscent of zebra

stripes, while cells infected with AdHrt2 failed to undergo organization of the sarcomere, with non-polymerized actinin remaining in “bundles”, similar to untreated conditions. When using antibodies to ANF, I was unable to determine a difference of ANF expression among any of the conditions, including no treatment and the addition of PE in the absence of virus, and therefore, was unable to make any conclusions about the effect on ANF by Hrt2. These observations were consistent with findings by light microscopy that cells in the presence of Hrt2 beat less frequently than control cell populations. These data suggest that AdHrt2 may block morphological changes of cardiomyocyte hypertrophy. Although an MOI over 3 pfu/cell is predicted by Poisson distribution to infect nearly all of the cells (Gerard and Meidell, 1995), the experiment can be improved by co-staining with anti-myc antibody to determine the effect of overexpression of Hrt2 on sarcomeric organization in each infected cardiomyocyte.

To determine if overexpression of Hrt2 could alter the induction of the fetal gene program during cardiomyocyte hypertrophy, I examined the expression of several markers of hypertrophy and the activation of the fetal cardiac gene program, including *ANF,* α*-MyHC,* β*-MyHc,* α*-skeletal actin* (Figure 1B). Hrt2 was able to attenuate the expression of *ANF* to basal levels in the presence or absence of PE. A similar expression profile was observed among all cardiac markers tested, although Hrt2 had little effect on the expression of the ubiquitous *GAPDH*. Taken together, these data suggest that overexpression of Hrt2 can repress morphological and

molecular characteristics observed during cardiomyocyte hypertrophy.

Hrt2 represses Gata4-mediated transactivation

To determine the molecular mechanism by which Hrt2 functions, a cadre of candidate cardiac transcription factors that regulate the fetal gene program were screened for sensitivity to Hrt-mediated repression and interaction with Hrt2. Of the 22 proteins tested on well-characterized cardiac promoters upstream of a luciferase reporter, Hrt2 was able to repress Gata4-mediated transactivation, one such factor, but not transactivation by certain other transcription factors, including SRF, Myocardin, dHAND, NFAT3 and MEF2C (Figure 2A and data not shown). Since Gata4 has been shown to cooperate with co-factors to increase *ANF*, I asked if Hrt2 could repress transactivation by Gata4 in conjunction with serum response factor (SRF), a GATA co-activator. I observed that the addition of Hrt2 was able to reduce Gata4-SRF transactivation to activity similar to the contribution by SRF (Figure 2A). This evidence implies that Hrt2 inhibits the action of these complexes, likely via Gata4, and that the components of these transactivation complexes can likely be separable.

I extended this analysis to other GATA-dependent enhancers, such as α-*MyHC* and *Nkx2.5* and observed a similar pattern (Figure 2B). In addition, I observed that the human orthologs of the Hrt family, HRT1, HRT2 and HRT3 were able to repress Gata4-dependent transactivation and found that Hrt2 repressed

other GATA family members, including Gata5 and Gata6 (Figure 2C, D). Thus, this evidence implies that Hrt-mediated repression of Gata4 transactivation is likely GATA-dependent.

Interaction is necessary for Hrt-mediated repression of Gata4

To determine if Hrt2 interacts with Gata4, I co-expressed FLAG-Gata4 and myc-Hrt2 in COS1 cells and observed that after immunoprecipitation with an anti-myc antibody, Gata4 was able to co-immunoprecipitate with Hrt2 (Figure 3A). To elucidate the GATA-interacting region of Hrt2, I incubated myc-Gata4 that was overexpressed in COS1 cells with radiolabeled proteins encoding Hrt2 mutations, constructed by I. King and O. Nakagawa, and performed an immunoprecipitation with anti-myc antibody (Figure 3B). Since the expression of various Hrt2 mutations was highly variable when overexpressed in COS1 cells, I synthesized ^{35}S-labeled protein products from rabbit reticulocyte lysate. In vitro binding analysis using C-terminal deletions of Hrt2 revealed that the Hrt2(2-207) mutation of Hrt2 was sufficient for interaction with Gata4 (Figure 3B). Smaller constructs, such as those encoding the bHLH region were poorly expressed in mammalian cells and results were difficult to analyze. To determine which regions within this mutation of Hrt2 were necessary for interaction, I tested several mutants with deletions of domains within the mutation of Hrt2(2-207) (Figure 3B). Even though mutations harboring a deletions of the orange domain or the loop segment bound to Gata4, I observed that

the mutation with the deletion of the basic motif, Δ(33-37), showed decreased binding affinity for Gata4 relative to other mutations (Figure 3B), implying that this region might be necessary for interaction with Gata4. Since these mutations were generated by in vitro transcription and translation, alterations of subcellular localization by deletion of the nuclear localization signal in the basic motif were avoided. These results were consistent with findings by us and others that the bHLH motif likely plays a critical role for mediating protein-protein interactions with Hrt co-factors (Iso et al., 2001b; Takata and Ishikawa, 2003; King et al., unpublished).

To determine if interaction of Hrt2 with Gata4 was necessary for Hrt-mediated repression of Gata4, I examined the effects of various mutants of Hrt2 on Gata4-dependent transactivation of *ANF*-luc (Figure 3C). Although most mutants were able to repress GATA-dependent transactivation, I observed that the mutation that failed to interact with Gata4, deletion of the basic domain, Δ(33-37), failed to repress GATA-mediated transactivation, compared to equivalent levels of wild-type Hrt2 protein expression (Figure 3C, D and data not shown). Since the basic motif acts as the nuclear localization signal (NLS), it is possible that the redistribution from nucleus to whole cell may also contribute to the loss of repressive activity in addition to the loss of binding to Gata4.

For determination of the Hrt2-interacting region of Gata4, I overexpressed myc-tagged mutations of Gata4 generated by C.L. Lien of the Olson lab in COS1 cells and incubated them with synthesized ^{35}S-labeled protein products from rabbit

reticulocyte lysate encoding FLAG-Hrt2 (Figure 4A). This method provided an alternative to co-immunoprecipitation in COS1 cells, since the mutations of Gata4 affected the expression of Hrt2 when co-expressed. Immunoprecipitation with anti-myc antibody and SDS-PAGE analysis revealed that the CZf and the NZf were sufficient for interaction with Hrt2. Deletion of the NZf, Gata4(240-332), revealed that this motif was not necessary for Hrt2 binding (Figure 4A, B). However, I was unable to efficiently express a mutation of Gata4 that lacked the CZf in COS1, precluding any analysis for the necessity of the CZf for the Hrt2-Gata4 interaction. I tried the converse experiment, to overexpress Hrt2 in COS1 cells and express Gata4 deletions in vitro, to circumvent the variable expression, particularly of the Gata4 mutant lacking the CZf, but I could not distinguish a difference in band intensity between the negative control and the experimental lane--a common problem with using radiolabeled proteins from reticulocyte lysate. As we recently found a disease-causing mutation of GATA4, Gata4(G295S), that affects the function of the CZf (Garg et al., 2003; Part 1), I tested if Hrt2 could interact with Gata4(G295S) and observed that Hrt2 was able to co-immunoprecipitate with Gata4(G295S), similar to wild-type Gata4 (data not shown). Consistent with this observation, Hrt2 was able to repress Gata4(G295S) transactivation of several GATA-dependent reporters (data not shown), suggesting that glycine 295 of Gata4 is not necessary for Hrt2 interaction or repression.

As Hrt2 interacts with the CZf adjacent to the NLS of Gata4, I asked if Hrt2

might affect the function of these motifs. Since the NLS is necessary for nuclear localization (Morrisey et al., 1997), I. King tested if overexpression of Hrt2 could affect the localization of Gata4. In HeLa cells, she found that Hrt2 did not alter the nuclear localization of Gata4 (data not shown). As the CZf is necessary for DNA binding, I tested if Hrt2 could affect DNA binding affinity of Gata4. In electromobility shift assays, I observed that increasing amounts of Hrt2 (up to 4 fold) did not affect DNA binding affinity of Gata4 to a radiolabeled GATA element (data not shown). As repression by Hrt2 of Gata4 is dependent on the interaction between Hrt2 and Gata4, it will be interesting to test by chromatin immunoprecipitation if Hrt2 recruits co-repressors, such as class I or III HDACs (Iso et al., 2001b; Takata and Ishikawa, 2003), to GATA-dependent promoters in cardiomyocytes.

Hrt-mediated repression of Gata4 is responsive to Akt

Although Hrt2 can repress Gata4-mediated transactivation of genes implicated in growth and differentiation, both are co-expressed in the developing embryonic heart and the hypertrophied heart at times when the heart is actively growing. To resolve this discrepancy, I speculated that a kinase or phosphatase that transmits a growth signal from the cell membrane to the nucleus might alter Hrt-mediated repression of Gata4. A cohort of nearly twenty kinases and phosphatases implicated in cardiac development and hypertrophy were tested for their effects on Hrt2-mediated repression of Gata4-dependent transactivation of *ANF* or α*-MyHC*-luc

(Figure 5A and data not shown). I observed only one, a constitutively active form of Akt/PKB (Akt*), which was able to ameliorate Hrt-mediated repression of transactivation by Gata4 (Figure 5A). Several kinases that were tested, including constitutively active forms of RSK2/MAPAPK and CaMKIV and wild type PKA, shared similar specificity for substrates (RXRXXS/T) as Akt, but they did not display a similar pattern (Figure 5A), suggesting that the responsiveness to Akt by Hrt-mediated repression of Gata4-dependent transactivation appeared to be specific to Akt.

Subsequent experiments were performed in collaboration with Dr. I. King. At low concentrations, Akt had little effect on Gata4 transactivation or Hrt2 repression of basal transactivation alone (Figure 5B), suggesting that the effect by Akt could not be attributed to either Gata4 or Hrt2. To determine if responsiveness to Akt by Hrt2 and Gata4 was mediated by Akt inhibition of GSK3 (Morisco et al., 2001), we tested a dominant negative form of GSK3b (dnGSK3b) (He et al., 1994) in the reporter assay, and we observed that Hrt2 was able to repress any increase in Gata4 transactivation by dnGSK3b (Figure 5B), suggesting that Akt-mediated amelioration of Hrt-mediated repression of Gata4-dependent transactivation was independent of GSK3 signaling.

Hrt2 can also repress Notch, a widely expressed transcription factor (Nakagawa et al., 1999). To determine if Akt could affect Hrt-mediated repression of other Hrt-sensitive transactivators, we compared the effect of Akt on Hrt2-mediated

repression of Gata4 transactivity of α-*MyHC*-luc and Hrt2-mediated repression of Notch-dependent transactivation of *Hrt2*-luc (Figure 5C, D). We observed that Akt did not affect Hrt-mediated repression of Notch, suggesting that the effect of Akt did not affect all targets of Hrt2-mediated repression.

Responsiveness to Akt is mediated by the orange domain of Hrt2

To determine if the effect of Akt was via Hrt proteins, we tested the responsiveness to Akt of several mutants of Hrt2 that could both interact and repress Gata4. We noticed that C-terminal deletion mutations containing the orange domain remained responsive to Akt, while deletion of the orange domain showed little responsiveness to Akt (Figure 6A), implying that the orange domain may mediate the effect by Akt.

We examined the amino acid sequence of the orange domain for serines, threonines and tyrosines, as potential phosphorylation sites, that were conserved across most Hrt family members. We found ten residues that matched these criteria, with one fitting the canonical consensus of Akt, RXRXXS/T, and two others loosely fitting this consensus (Figure 6B). We tested mutations of serine or threonine residues in this domain of Hrt2 that were conserved among all three Hrt family members in the reporter assay. Mutation of serine 155 to glutamic acid (S155E) mimicked the effects of Akt on Hrt-mediated repression of Gata4 transactivation (Figure 6C). Likewise, we observed that Hrt2 (S155E) was able to

repress Notch-mediated transactivation (Figure 6D), suggesting that the mutant might function as a mimic of the Akt.signal.

It should be noted that I. King has generated several mutations of serine to alanine in the orange domain, including S155A, but no single mutation is resistant to the Akt signal, suggesting that more than one serine may be involved. She is now generating combinations of Ser or Thr to Ala mutants of Hrt2 and testing their responsiveness to Akt. However, it still remains to be determined if Hrt2 can be phosphorylated by Akt. An intermediate step or an Akt-related kinase may mediate the effect on the orange domain of Hrt2. Alternatively, Gata4 may be the target of the Akt signal and is therefore being tested as a substrate in the in vitro kinase assay. This possibility remains less likely, since we have rarely observed increases in Gata4-dependent transactivation by Akt in HeLa cells,

Mechanism of action by Akt on Hrt and Gata remains unknown

Since Akt is known to affect the nuclear localization of forkhead transcription factors (reviewed in Kops and Burgering, 1999), we asked if Akt could affect the localization of Hrt2 or Gata4. I. King observed that Akt did not affect nuclear localization of Hrt2 or Gata4 in HeLa or COS1 cells nor did she observe alterations in nuclear localization by Hrt2(S155E), consistent with a mechanism likely other than changes in subcellular localization.

Akt has also been shown to disrupt the interaction between transcriptional co-

factors (Puigserver et al., 2003). Therefore, I tested if the addition of Akt could disrupt the interaction between Gata4 and Hrt2. Using co-immunoprecipitation assays in COS1 cells, I did not observe a change in the interaction between Gata4 and Hrt2 in the presence of Akt (data not shown). However, it remains difficult to assess if the amount of constitutively active Akt was sufficient for the effect observed in reporter assays, since GATA-dependent luciferase assays display little activation when performed in COS1 cells. In an attempt to circumvent this issue, I used Hrt2(S155E) as a mimic of the Akt signal and observed that Hrt2(S15E) could still interact with Gata4 to the same degree as wild type Hrt2 (data not shown). To examine the effect of the Akt signal on the recruitment of co-repressors by Hrt2, I tested if the co-repressors HDAC1 and HDAC3 could co-immunoprecipitate with Hrt2(S155E). I observed an interaction between HDAC1 or HDAC3 and Hrt2(S155E), similar to wild type Hrt2 (data not shown), consistent with a hypothesis that the Akt signal may affect a subset of Hrt2 interactions or functions. To help explain the specificity of the effect by Akt on Hrt2 and Gata4, but not Hrt2 and Notch, we are testing if an interaction between Hrt2 and other GATA co-factors, such as Fog-2, might be disrupted by the Akt signal. As well, we are testing the effects of Akt on Fog-mediated repression of Gata4 in the presence or absence of Hrt2.

DISCUSSION

This work provides evidence for a molecular pathway by which growth signals, via a second messenger, can affect gene expression. Here, I showed that overexpression of Hrt2 repressed endogenous cardiac gene expression in cardiomyocyte hypertrophy. Hrt2 specifically repressed GATA-dependent transactivation via an interaction with Gata4. Finally, we found that Hrt-mediated repression of Gata4 transactivation is regulated by an Akt-dependent signal. Together, this study provides a step into understanding how the cardiomyocyte might respond to extracellular signals.

Several questions remain. It will be important to ascertain if Hrt2 can repress various agonists that are mediated by different signaling cascades, as sensitivity to Hrt-mediated repression may elucidate some specificity of Hrt2 to particular pathways. As well, it will be necessary to demonstrate that overexpression of Akt in cardiomyocytes can overcome Hrt2-mediated repression of *ANF*, to strengthen the evidence for the effect of Akt on Hrt-mediated repression of GATA-dependent transcription using luciferase assays in non-cardiac cells. Since Hrt2 is phosphorylated in cardiomyocytes when overexpressed, it will be necessary to determine if this phosphorylation is mediated by Akt, by overexpressing a dominant negative form of Akt and examining the phosphorylation status of Hrt2 by western analysis. The effect of overexpression of mutants of Hrt2, particularly Δ(120-164) that are not responsive to Akt and the S155E mutation that acts as a mimic of Akt,

will be tested, to determine if the orange domain acts as an Akt-responsive domain of Hrt2 in cardiomyocytes. Akt-dependent phosphorylation and mapping of Hrt2 remains critical as an entry point to detemining the mechanism by which Akt might affect Hrt2 and Gata4. In addition, it should be determined if other GATA co-repressors, such as Fog-2, are responsive to Akt as a common mechanism for regulating GATA-dependent transcription, or if inhibition of other GATA co-repressors may be disrupted in the presence of Hrt2 and Akt.

More importantly, we do not yet know if Hrt2 plays an in vivo role to modulate cardiac hypetrophy. To do so, Osamu Nakagawa will be testing if mice heterozygous for *Hrt2* display increased sensitivity to various hypertrophic stimuli, including: (1) thoracic aortic banding (Rockman et al. 1991); (2) a cardiac hypertrophy transgenic mouse model that drives a constitutively active form of CnA in the heart (Molkentin et al., 1997); (3) an isoproterenol pump (Kudej et al., 1997); or (4) intraperitoneal injection of IGF-1 (Shioi et al., 1997). He will also test if Hrt2 represses GATA function in vivo, by examining if loss of Hrt2 can rescue embryonic lethality of mice harboring heterozygote alleles of Gata4 and Gata6 (Davis, Nakagawa and colleagues, unpublished). All of these experiments are underway.

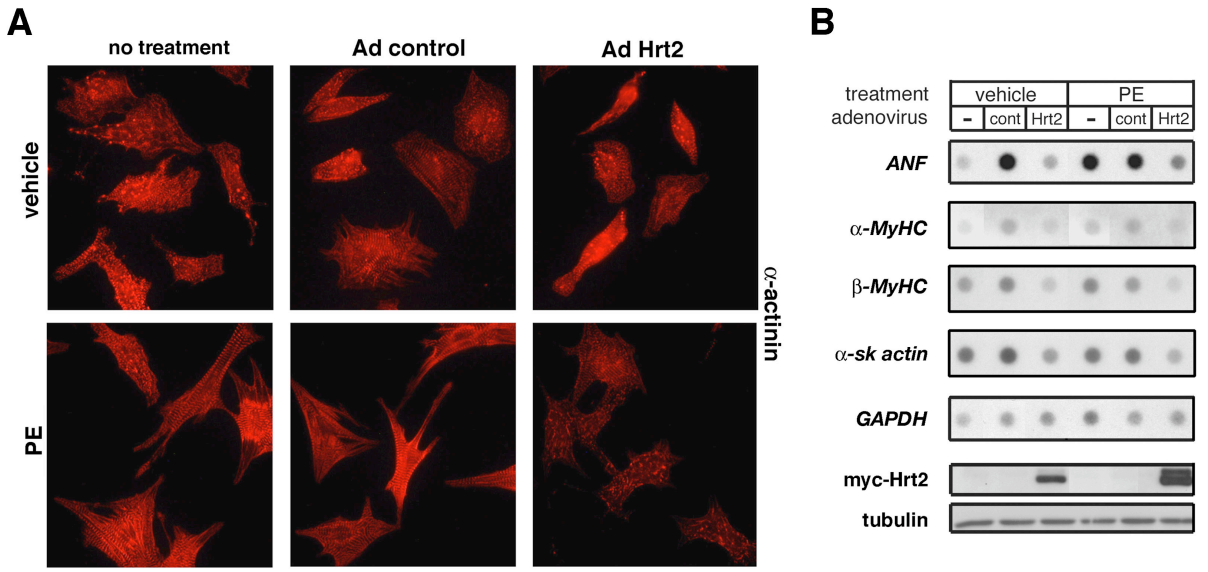


Figure 1. Hrt2 inhibits hallmarks of cardiomyocyte hypertrophy. (A) Neonatal rat cardiomyocytes were cultured in the absence of virus or were infected with adenoviruses encoding viral machinery (Adcontrol) or myc-tagged Hrt2 at a multiplicity of infection (MOI) of 30 pfu/cell. After stimulation with PE for 24 hours, cells were fixed and stained with antibody against sarcomeric α-actinin (red signal). (B) RNA from cardiomyocytes under each condition was analyzed for transcripts of *ANF, α-MyHC, β-MyHC* and *α-skeletal actin* by dot blot analysis. *GAPDH* was used as loading control. The expression of ectopic myc-Hrt2 in cardiomyocyte lysates was determined by Western analysis of 0.2% of total cell lysate, using an anti-myc antibody for detection of overexpressed Hrt2, or tubulin as a loading control. At least two independent experiments were performed, with representative data shown. PE, phenylephrine; cont, control; no treatment indicated by (-); ANF, atrial natriuretic factor; α-MyHC, alpha myosin heavy chain; β-MyHC, beta myosin heavy chain; α-sk actin, α-skeletal actin; GAPDH, glycerol-3-phosphate dehydrogenase.

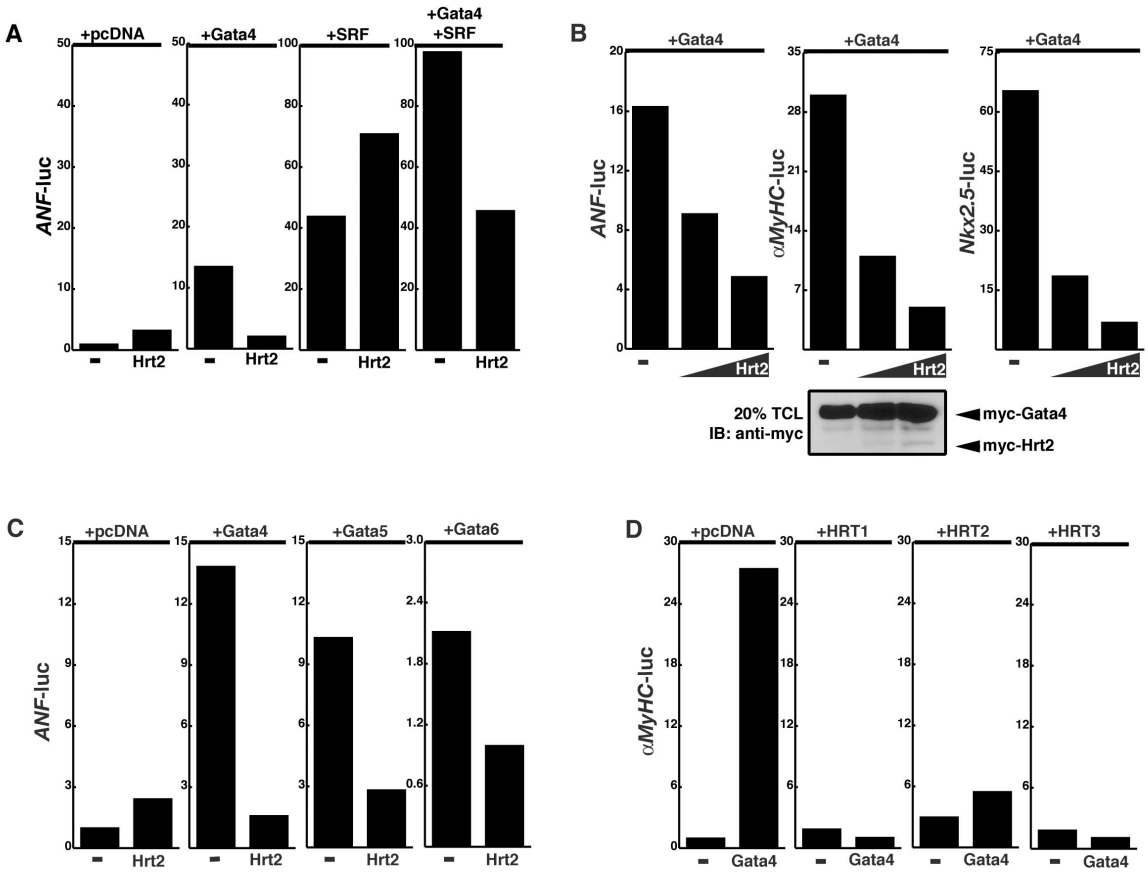


Figure 2. Hairy-related transcription factors repress GATA-dependent transactivation. (A) In HeLa cells, pcDNA or Hrt2 was co-expressed with several cardiac transcription factors, including Gata4 and SRF, and transactivation of a cardiac promoter upstream of a luciferase reporter, *ANF*-luc, was assayed. (B) The effect of increasing amounts of Hrt2 on the activity of Gata4 on various GATA-dependent promoters, *ANF*-luc, α-*MyHC*--luc and *Nkx2.5*-luc, was tested. (C) Transactivation by cardiac members of the GATA family, Gata4, Gata5 and Gata6, on *ANF*-luc was determined in the presence or absence of Hrt2. (D) Luciferase activity directed by the *ANF* promoter in the presence of Gata4 and human orthologs of the Hrt family, HRT1, HRT2, and HRT3. At least two independent experiments were performed, with representative data shown. Fold activation above baseline is depicted. TCL, total cell lysate; IB, immunoblot.

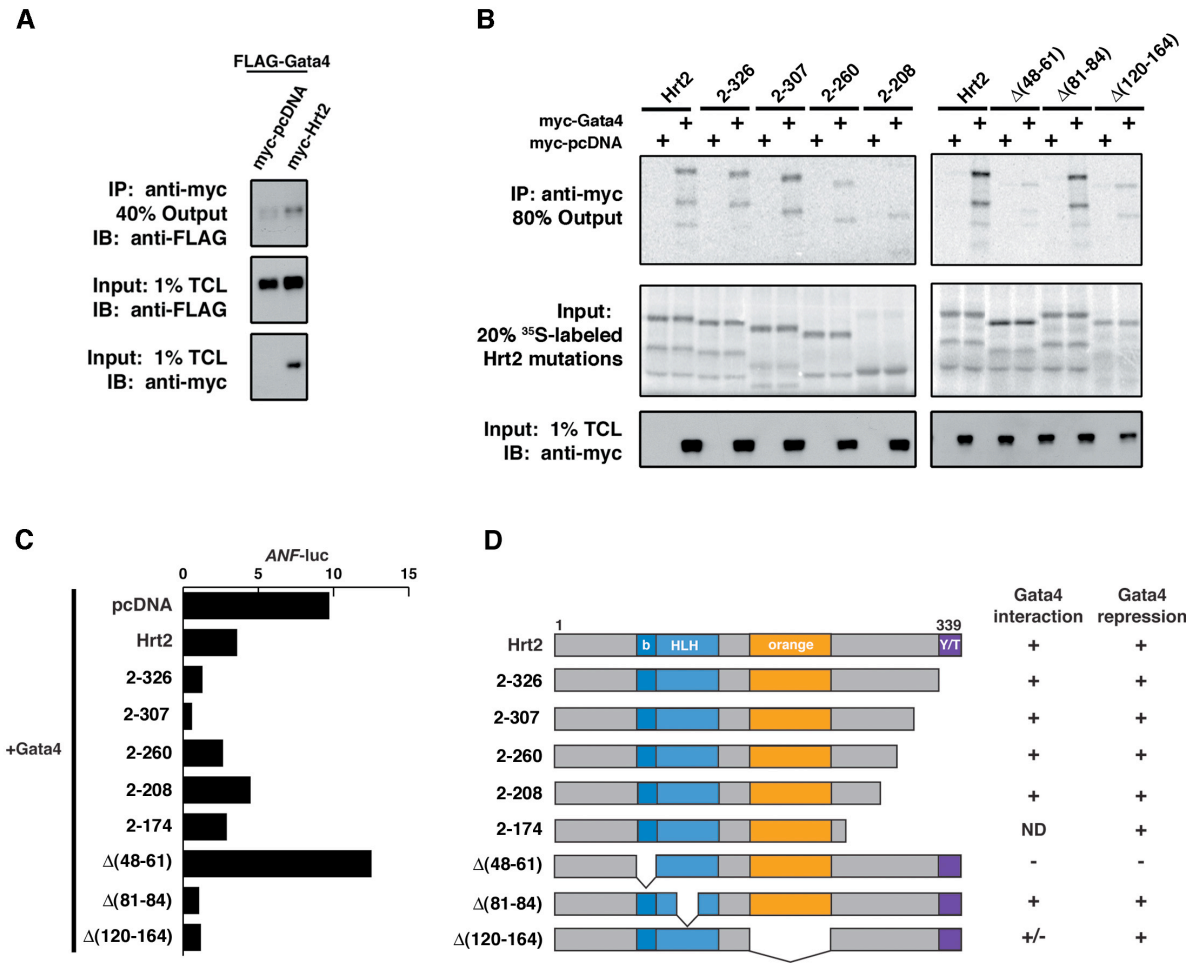


Figure 3. Interaction of Hrt2 with Gata4 is necessary for Hrt-mediated repression. (A) FLAG-Gata4 was overexpressed with myc-Hrt2 or myc-pcDNA in COS1 cells and protein lysates were subjected to immunoprecipitation (IP) with anti-myc antibody. Subsequent Western analysis using anti-myc antibody on 40% of output, and anti-myc or anti-FLAG antibody on 1% of input are shown. (B) Radiolabeled C-terminal mutations of Hrt2 were incubated with COS1 lysate overexpressing myc-Gata4 and following immunoprecipitation (IP) with anti-myc antibody, output was loaded on SDS-PAGE and subjected to phosphoimage analysis. Input of ^{35}S-labeled Hrt2 mutations proteins and overexpression of myc-Gata4 or myc-pcDNA in cell lysates are shown. Radiolabeled mutations of domains within del(208-339) of Hrt2 were incubated with myc-Gata4 or myc pcDNA that was overexpressed in COS1 cells and subjected to the in vitro binding assay, as above. (C) The effect of mutations of Hrt2 on Gata4-dependent transactivation of *ANF*-luc reporter was assessed. At least two independent experiments were performed, with

representative data shown. Fold activation above baseline is depicted. (D) Schematic and summary of the effect of Hrt2 mutations on interaction or repression of Gata4. Functional domains are highlighted; basic (b) motif in dark blue, helix-loop-helix (HLH) in light blue, orange domain in orange, and C-terminal YRPW/TEIGAF (Y/T) in purple. TCL, total cell lysate; IB, immunoblot.

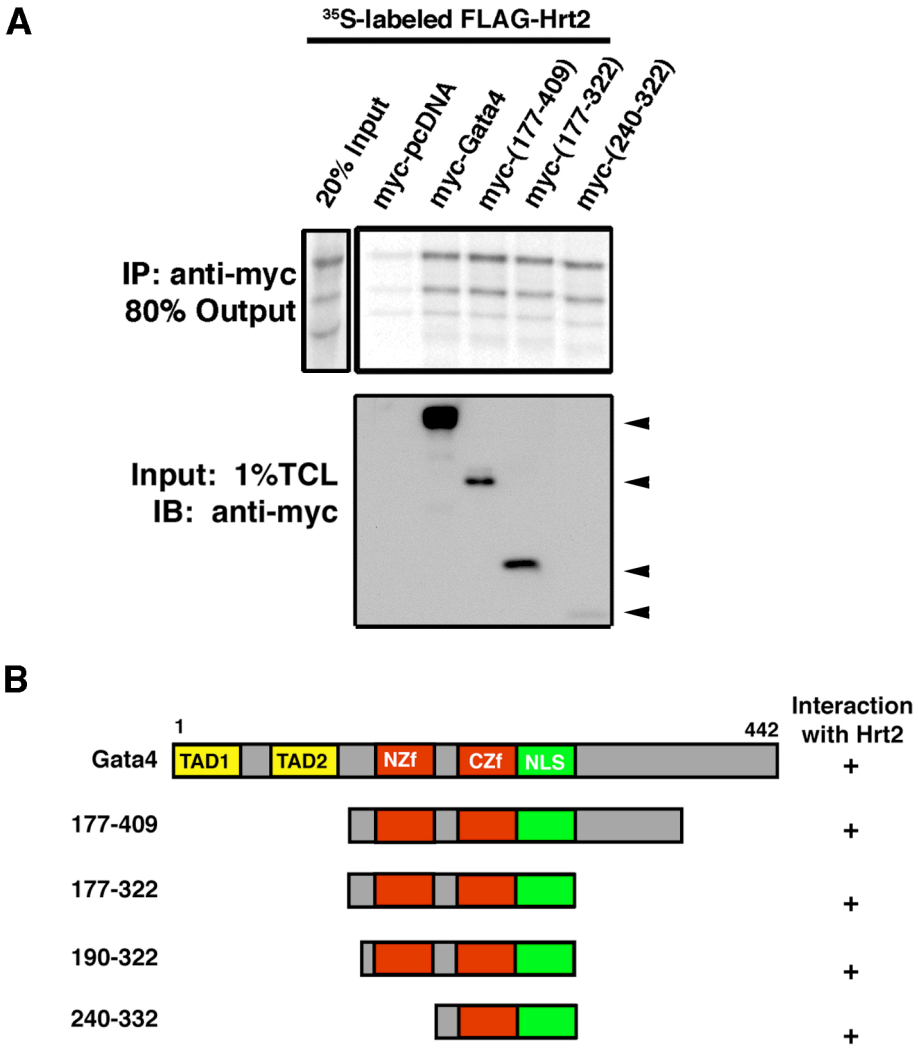


Figure 4. Hrt2 interacts with the C-terminal zinc finger and nuclear localization signal of Gata4. (A) Overexpressed myc-tagged deletions of Gata4 in COS1 cells were incubated with radiolabeled Hrt2 and immunoprecipitated with anti-myc antibody. Following SDS-PAGE, output was exposed to phosphoimage analysis. (B) Schematic and summary of interaction between Hrt2 and mutations of Gata4. Domains are highlighted; bipartite transactivation domain (TAD1 and TAD2) in yellow, N-terminal (NZf) and C-terminal (CZf) zinc-finger motifs in red, and nuclear localization signal (NLS) in green. TCL, total cell lysate; IB, immunoblot.

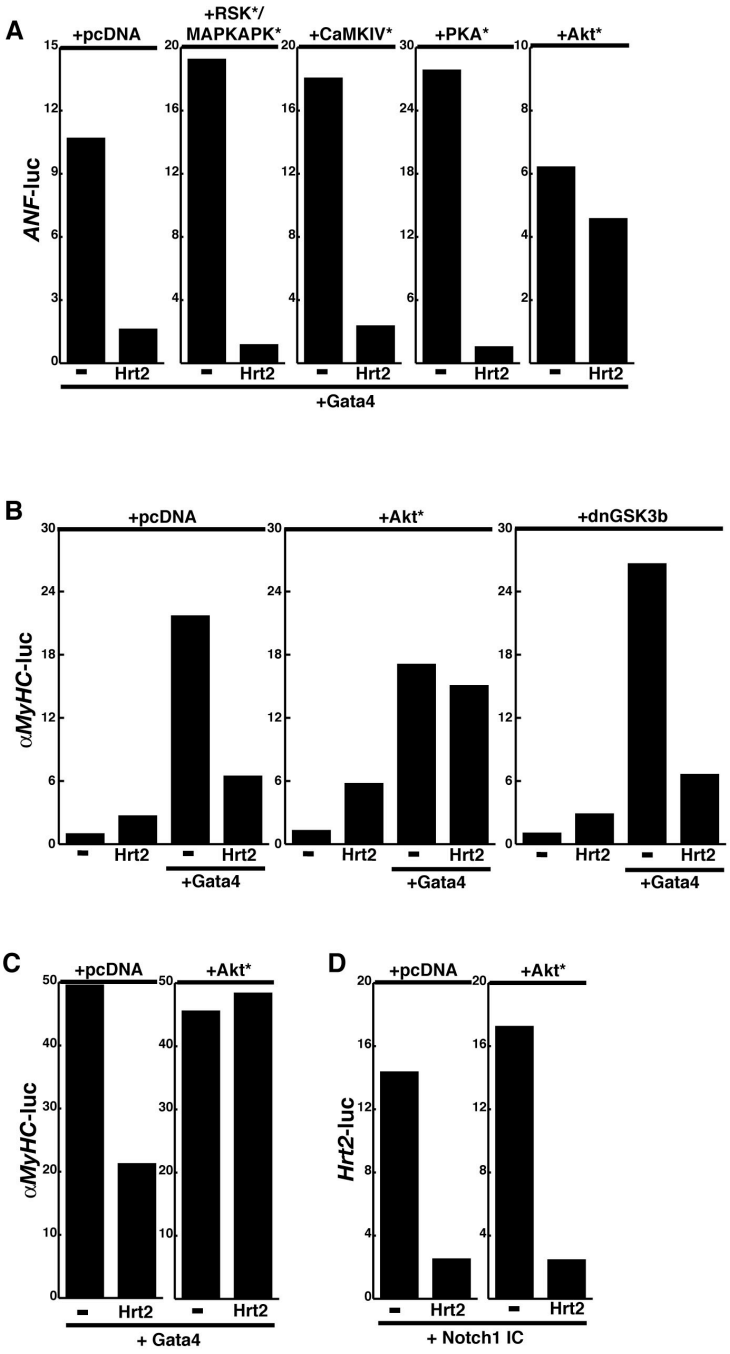


Figure 5. Hrt2-mediated repression of Gata4-dependent transactivation is sensitive to Akt. (A) In HeLa cells, disruption of Hrt2-mediated repression of Gata4-dependent transactivation of the *ANF*-luc reporter was tested in the presence of several kinases with similar substrate specificity, including constitutively active forms of RSK2, also known as MAPKAPK (RSK2*/MAPKAPK*), CaMKIV (CaMKIV*) and Akt, also known as PKB (Akt*/PKB*), and wild-type PKA. (B) Lesser amounts of Akt* were assayed for responsiveness of Hrt-mediated repression of Gata4 transactivation of α-*MyHC*-luc reporter. Since inhibition of GSK3b may mediate the Akt signal, the effect by overexpression of a dominant negative form of GSK3b (dnGSK3b) on Hrt2-mediated repression of Gata4-dependent transactivation of α-*MyHC*-luc was determined. (C) In the presence or absence of Akt*, Hrt2-mediated repression of Gata4 transactivation of *ANF-luc* reporter was compared to Hrt2-mediated inhibition of Notch transactivation of *Hrt2*-luc reporter (D). At least two independent experiments were performed, with representative data shown. Fold activation above baseline is depicted.

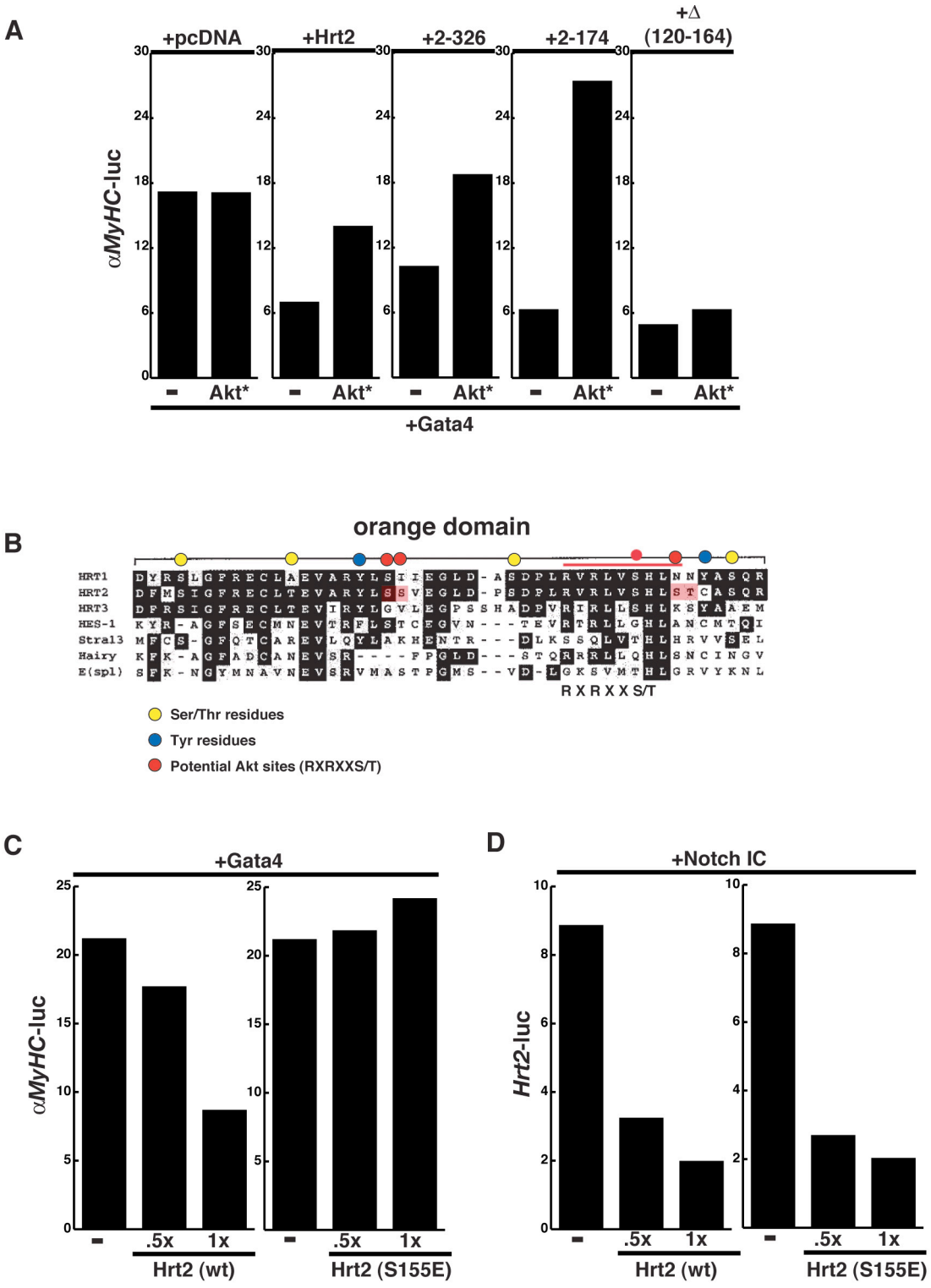

A
+pcDNA
+Hrt2
+2-326
+2-174
+Δ (120-164)
αMyHC-luc
0
6
12
18
24
30
-
Akt*
+Gata4
B
orange domain
HRT1
HRT2
HRT3
HES-1
Stral3
Hairy
E(spl)
R X R X X S/T
Ser/Thr residues
Tyr residues
Potential Akt sites (RXRXXS/T)
C
+Gata4
αMyHC-luc
.5x
1x
Hrt2 (wt)
Hrt2 (S155E)
D
+Notch IC
Hrt2-luc

Figure 6. The orange domain of Hrt2 is responsive to Akt. (A) Represssion of Gata4-dependent transactivation of α-*MyHC*-luc reporter by C-terminal mutations of Hrt2 was assessed for responsiveness to constitutively active Akt (Akt*) in HeLa cells. (B) Positions of conserved Ser/Thr/Tyr in the amino acid sequence of the orange domain of Hrt family members. Canonical consensus sites for Akt phosphorylation are indicated as red circles. Yellow circles highlight conserved Ser/Thr residues, and blue circles denote conserved Tyr residues. (C) Point mutation of serine 155 to glutamic acid, Hrt2(S155E), was tested for its effect on Gata4-dependent transactivation of α-*MyHC*-luc reporter. (D) Luciferase activity driven by Notch-dependent transactivation of *Hrt2*-luc, in the presence of wild-type Hrt2 or Hrt2(S155E). At least two independent experiments were performed, with representative data shown. Fold activation above baseline is depicted. wt, wild-type.

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VITAE

Irfan S. Kathiriya was born in Detroit, Michigan on May 13, 1975, to Drs. Sugra S. Kathiriya, his mother, a pediatrician, and Shiraz A. Kathiriya, his father, a chemist, of Indian descent. Irfan graduated from Sunny Hills High School in southern California, with an International Baccalaureate degree as a three sport Varsity letterman. While attending Claremont McKenna College, he pursued his varied interests in government and science, and graduated summa cum laude with a Bachelor of Arts degree in Chemistry with Honors and International Relations, with recognition as Scholastic All-America in Track and Field. To develop as a physician-scientist, he moved to Dallas, TX, to attend the University of Texas Southwestern Medical School as a fellow of the Medical Scientist Training Program. Against all odds, he met his wife, Isabelle King, M.D., in the lab, proving once again that there is someone for everyone. Following graduation, Irfan is moving to the Bay Area, to pursue clinical training in anesthesia and research training in the neurosciences at the University of California, San Francisco.

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